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Original Article

A regulatory role of androgen in ovarian steroidogenesis by rat granulosa cells

Toru Hasegawa[a], Yasuhiko Kamada[a], Takeshi Hosoya[b], Shiho Fujita[a], Yuki Nishiyama[b], Nahoko Iwata[c], Yuji Hiramatsu[a], Fumio Otsuka[*,c]

[a] Departments of Obstetrics Gynecology
[b] Medicine and Clinical Science
[c] General Medicine, Okayama University Graduate School of Medicine, Dentistry Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kitaku, Okayama, 700-8558, Japan

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ABSTRACT

Excess androgen and insulin-like growth factor (IGF)-I in the ovarian follicle has been suggested to be involved in the pathophysiology of polycystic ovary syndrome (PCOS). Here we investigated the impact of androgen and IGF-I on the regulatory mechanism of ovarian steroidogenesis using rat primary granulosa cells. It was revealed that androgen treatment with dihydrotestosterone (DHT) amplified progesterone synthesis in the presence of FSH and IGF-I, whereas it had no significant effect on estrogen synthesis by rat granulosa cells. In accordance with the effects of androgen on steroidogenesis, DHT enhanced the expression of progesterogenic factors and enzymes, including StAR, P450scc and 3βHSD, and cellular cAMP synthesis induced by FSH and IGF-I. Of note, treatment with DHT and IGF-I suppressed Smad1/5/8 phosphorylation and transcription of the BMP target gene Id-1, suggesting that androgen and IGF-I counteract BMP signaling that inhibits FSH-induced progesterone synthesis in rat granulosa cells. DHT was revealed to suppress the expression of BMP-6 receptors, consisting of ALK-2, ALK-6 and ActRII, while it increased the expression of inhibitory Smads in rat granulosa cells. In addition, IGF-I treatment upregulated androgen receptor (AR) expression and DHT treatment suppressed IGF-I receptor expression on rat granulosa cells. Collectively, the results indicate that androgen and IGF-I mutually interact and accelerate progesterone production, at least in part, by regulating endogenous BMP signaling in rat granulosa cells. Cooperative effects of androgen and IGF-I counteract endogenous BMP-6 activity in rat granulosa cells, which is likely to be functionally linked to the steroidogenic property shown in the PCOS ovary.

1. Introduction

In patients with polycystic ovary syndrome (PCOS), endogenous insulin levels are increased due to insulin resistance [1]. Hyperinsulinemia reinforces local insulin-like growth factor (IGF)-I activity in the ovary and, as a result, this induces an androgenic composition in theca cells [2]. Both increased IGF-I activity and a high androgenic environment appear to be involved in the ovulation disorder in PCOS patients [3].

The growing and maturating process of ovarian follicles occurs as a consequence of functional interactions between gonadotropins and various autocrine/paracrine factors in the ovary. It has been shown that the ovarian bone morphogenetic protein (BMP) system plays a critical role in the regulation of follicular steroidogenesis in an autocrine/paracrine manner [4,5]. BMPs exert a common biological activity to suppress progesterone synthesis stimulated by follicle-stimulating hormone (FSH) as a luteinizing inhibitor. In growing follicles, exquisite control of FSH responsiveness is necessary for the physiological selection of dominant follicles and the successive ovulation. BMPs are key regulatory molecules for the maintenance of FSH activity as well as FSH sensitivity during the process of normal folliculogenesis [4,6].

The BMP system molecules including BMP ligands and the receptors exhibit a cell-specific expression pattern in ovarian follicles. Among these, the expression of BMP-6 is localized in oocytes and granulosa

Abbreviations: ALK, activin receptor-like kinase; ActRI, activin type-I receptor; ActRII, activin type-II receptor; BMP, bone morphogenetic protein; BMPRI, BMP type-I receptor; BMPRII, BMP type-II receptor; FSH, follicle-stimulating hormone; FSHR, FSH receptor; GDF, growth and differentiation factor; IGF-I, insulin-like growth factor-I; IGF-IR, IGF-I receptor; 3βHSD, 3β-hydroxysteroid dehydrogenase; LH, luteinizing hormone; PCOS, polycystic ovary syndrome; P450arom, P450 aromatase; P450scc, P450 steroid side-chain cleavage enzyme; StAR, steroidogenic acute regulatory protein; TGF, transforming growth factor

* Corresponding author at: Department of General Medicine, Okayama University of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata－cho, Kitaku, Okayama 700－8558, Japan.
E-mail address: fumiotsu@md.okayama-u.ac.jp (F. Otsuka).

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cells at Graafian stages of rat healthy follicles [6]. BMP-6 has an inhibitory effect on FSH receptor actions by suppressing adenylate cyclase, leading to cAMP reduction, in rat granulosa cells [7,8]. BMP-6 expressed in rat granulosa cells appears to be reduced at the step of selecting dominant follicles [6], suggesting that BMP-6 physiologically conduces to the selection process.

From the standpoint of the pathophysiology of human PCOS, examination of ovary tissues revealed that growth and differentiation factor (GDF)-9 mRNA expression, compared to BMP-15, was delayed and decreased in growing follicles from PCOS ovaries [9]. Moreover, it is of note that the expression of BMP-6 is highly increased in human granulosa cells isolated from PCOS ovaries [10,11].

In the present study, we investigated the functional roles of androgen and IGF-I in ovarian steroidogenesis by focusing on BMP-6, which suppresses progesterone biosynthesis, using primary culture of rat granulosa cells. It was clarified that androgen excess and IGF-I action interact mutually and accelerate steroidogenesis by regulating endogenous BMP signaling in rat granulosa cells.

2. Materials and methods

2.1. Reagents and supplies

McCoy's 5A medium, Medium 199 and HEPES buffer solution were purchased from Invitrogen Corp. (Carlsbad, CA). Bovine serum albumin (BSA), diethylstilbestrol (DES), dihydrotestosterone (DHT), 4-androstene-3,17-dione, 3-isobutyl-1-methylxanthine (IBMX), insulin-like growth factor-I (IGF-I), ovine pituitary FSH, and penicillin-streptomycin solution were purchased from Sigma-Aldrich Co. Ltd. (St. Louis, MO). Recombinant human BMP-6 was purchased from R & D Systems Inc. (Minneapolis, MN).

2.2. Primary culture of rat ovarian granulosa cells

Female Sprague-Dawley (SD) rats were purchased from Charles River (Wilmington, MA). To increase the number of granulosa cells, silastic capsules containing 10 mg of DES were implanted in 22-day-old female SD rats. After 3 days of exposure to DES, ovarian follicles were punctured with a 27-gauge needle. Granulosa cells were counted and separated from oocytes by filtering the oocyte/granulosa cell suspension through 100- and 40-μm nylon meshes (BD Falcon, Bedford, MA) that allowed granulosa cells but not oocytes to pass through [12,13]. The isolated granulosa cells were then cultured in a serum-free McCoy's 5A medium supplemented with penicillin-streptomycin at 37 °C in an atmosphere of 5% CO_2. The animal protocols were approved by Okayama University Institutional Animal Care and Use Committee.

2.3. Determination of estradiol, progesterone and cAMP levels

Rat granulosa cells (1×10^5 viable cells in 200 μl) were cultured, as described above, in 96-well plates with 100 nM of androstenedione, a substrate for aromatase. FSH (10 ng/ml) was added to the culture medium either alone or in combination with indicated concentrations of IGF-I and DHT. After 48-h culture, the culture media were collected and stored at −30 °C until assay. The concentrations of estradiol and progesterone in the culture medium were examined by a chemiluminescent immunoassay (CLIA) using Architect estradiol and progesterone kits (Cayman Chemical Co., Ann Arbor, MI, USA). Steroid contents were undetectable (progesterone < 10 pg/ml and estradiol < 15 pg/ml) in cell-free medium. To evaluate cellular cAMP synthesis, rat granulosa cells (1×10^5 viable cells in 200 μl) were cultured, as described above, in 96-well plates with 0.1 mM of IBMX (specific inhibitor of phosphodiesterase activity). After 48-h culture with indicated treatments, the conditioned medium was collected and stored at −30 °C until assay. The concentrations of extracellular cAMP were measured by an enzyme-linked immunosorbent assay (ELISA; Enzo Life Sciences, Inc., NY, USA) after acetylation of each sample with assay sensitivity of 0.039 pmol/ml.

2.4. Granulosa cellular RNA extraction and quantitative RT-PCR

Rat granulosa cells (5×10^5 viable cells in 1 ml) were cultured in 12-well plates with serum-free McCoy's 5A. Cells were treated with FSH (10 ng/ml) either alone or in combination with indicated concentrations of IGF-I and DHT. After 48-h culture, total cellular RNA was extracted using TRIzol® (Invitrogen Corp.). Total RNA amount was quantified by measuring the absorbance of the sample at 260 nm, and stored at −80 °C until assay. Primer pairs for activin receptor-like kinase (ALK)-2, ALK-3 and ALK-6, activin type-II receptor (ActRII), BMP type-II receptor (BMPRII), Smad6 and 7, and ribosomal protein L19 (RPL19) were selected as reported previously [14,15]. For all of the genes, the primer pairs were selected from different exons of the corresponding genes to distinguish PCR products that might arise from chromosome DNA contaminants as follows: P450scc, 147-167 and 636-655 (from GenBank accession #J05156); steroidogenic acute regulatory protein (StAR), 395-415 and 703-723 (AB001349); rat type-I 3β-hydroxysteroid dehydrogenase (3βHSD), 336-355 and 841-860 (M38178); P450arom, 1180-1200 and 1461-1481 (M33986); Id-1, 225-247 and 364-384 (NM_010495); IGF-I receptor (IGF-IR), 1266-1287 and 1501-1522 (NM_052807); androgen receptor (AR), 2521-2543 and 2711-2734 (NM_013476); and RPL19, 401-421 and 575-595 (J02650). The extracted RNA (1 μg) was subjected to reverse transcription using a First-Strand cDNA Synthesis System (Invitrogen Corp.) with random hexamer (2 ng/μl), reverse transcriptase (200 U) and deoxynucleotide triphosphate (dNTP; 0.5 mM) at 42 °C for 50 min and at 70 °C for 10 min. To quantify the level of target gene mRNA, real-time PCR was performed using the LightCycler® Nano real-time PCR system (Roche Diagnostic Co., Tokyo, Japan) after optimization of annealing conditions. The relative mRNA expression was calculated by the Δ threshold cycle (Ct) method, in which ΔCt is the value obtained by subtracting the Ct value of RPL19 mRNA from the Ct value of the target mRNA, and the amount of target mRNA relative to RPL19 mRNA was expressed as $2^{-(\Delta Ct)}$. The data are expressed as the ratio of target mRNA to RPL19 mRNA.

2.5. Western immunoblotting analysis

Rat granulosa cells (2.5×10^5 viable cells in 500 μl) were cultured in 24-well plates in serum-free McCoy's 5A medium. After preculture either alone or with indicated concentrations of IGF-I and/or DHT, cells were treated with BMP-6 for 60 min. The cells were solubilized in 100 μl RIPA lysis buffer (Upstate Biotechnology, Lake Placid, NY) containing 1 mM Na_3VO_4, 1 mM NaF, 2% SDS, and 4% β-mercaptoethanol. The cell lysates were subjected to SDS-PAGE/immunoblotting analysis using anti-phospho-Smad1/5/8 (pSmad1/5/8) antibody (Cell Signaling Technology, Inc., Beverly, MA) and anti-actin antibody (Sigma-Aldrich Co. Ltd.). The integrated signal density of each protein band was analyzed by the C-DiGit® Blot Scanner System (LI-COR Biosciences, NE). To evaluate the target protein levels, the ratios of the signal intensities of target proteins/actin were calculated.

2.6. Statistical analysis

All results are shown as means ± SEM of data from at least three separate experiments, each performed with triplicate samples. Differences between groups were statistically analyzed using ANOVA or unpaired *t*-test, when appropriate (StatView 5.0 software, Abacus Concepts, Inc., Berkeley, CA). If differences were detected by ANOVA, Fisher's protected least significant difference (PLSD) test and Tukey-Kramer's post hoc test were used to determine which means differed (StatView 5.0 software). *P* values < 0.05 were accepted as statistically significant.