Original paper

Involvement of BMP-15 in glucocorticoid actions on ovarian steroidogenesis by rat granulosa cells.

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Running title: Glucocorticoid effects on ovarian steroidogenesis

***Key words*:** Bone morphogenetic protein (BMP), Glucocorticoid, Glucocorticoid receptor (GR), Granulosa cells, and Steroidogenesis

Disclosure Statement: The authors have nothing to disclose.

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Abbreviations:

ActRII, activin type-II receptor; Aldo, aldosterone; ALK, activin receptor-like kinase; 20αHSD, 20α-hydroxysteroid dehydrogenase; 3βHSD, 3β-hydroxysteroid dehydrogenase; BMP, bone morphogenetic protein; BMPRII, BMP type-II receptor; Dex, dexamethasone; FSH, follicle-stimulating hormone; FSHR, FSH receptor; GR, glucocorticoid receptor; HPO, hypothalamo–pituitary–ovarian; MR, mineralocorticoid receptor; PCOS, polycystic ovary syndrome; P450arom, P450 aromatase; P450scc, P450 steroid side-chain cleavage enzyme; RPL19, ribosomal protein L19; and StAR, steroidogenic acute regulatory protein.

Word counts: 4431 words, **Figure number:** 4 figures

Abstract

To elucidate the impact of glucocorticoids on ovarian steroidogenesis and its molecular mechanism by focusing on bone morphogenetic proteins (BMPs), we examined the effect of dexamethasone (Dex) on estradiol and progesterone synthesis by using primary culture of rat granulosa cells. It was revealed that Dex treatment dose-dependently decreased estradiol production but increased progesterone production induced by follicle-stimulating hormone (FSH) by granulosa cells. In accordance with the effects of Dex on estradiol synthesis, Dex suppressed P450arom mRNA expression and cAMP synthesis induced by FSH. Dex treatment in turn enhanced basal as well as FSH-induced levels of mRNAs encoding the enzymes for progesterone synthesis including P450scc and 3βHSD but not StAR and 20αHSD. Of note, Dex treatment significantly upregulated transcription of the BMP target gene Id-1 and Smad1/5/9 phosphorylation in the presence of BMP-15 among the key ovarian BMP ligands. It was also found that Dex treatment increased the expression level of BMP type-I receptor ALK-6 among the type-I and -II receptors for BMP-15. Inhibitory Smad6/7 expression was not affected by Dex treatment. On the other hand, BMP-15 treatment upregulated glucocorticoid receptor (GR) expression in

granulosa cells. Collectively, it was revealed that glucocorticoids elicit differential effects on ovarian steroidogenesis, in which GR and BMP-15 actions are mutually enhanced in granulosa cells.

Introduction

The glucocorticoid receptor (GR) is known to be expressed in the ovary and it has been shown that glucocorticoids exert direct effects on granulosa cell functions [1]. In a clinical setting, menstrual abnormality, amenorrhea and hypermenorrhea can be manifested in patients with glucocorticoid excess. On the other hand, glucocorticoids can also be used for treatment of polycystic ovary syndrome (PCOS) with hyperandrogenism [2]. However, the effects of glucocorticoids on the reproductive system have not been fully elucidated.

It has been shown that moderate levels of glucocorticoids can support female reproductive processes at the anterior pituitary level and may have an anti-inflammatory function in the ovary and adjacent tissues [1,3,4]. However, stress levels of glucocorticoids adversely affect hypothalamo–pituitary–ovarian (HPO) function in the adult females. Stress and glucocorticoids inhibit the secretion of gonadotropins in both genders [5,6], although this effect in males is

modified by inhibitory effects of glucocorticoids on the testes. Diminished ovarian response to gonadotropins such as glucocorticoid-induced inhibition of steroidogenesis by human granulosa-luteum cells [7] and inhibition of progesterone synthesis by rat preovulatory follicles [8] has been experimentally shown. However, glucocorticoid-induced suppression of the female reproductive system seems to be complex and cannot be simply explained by the interaction of glucocorticoids and GR [6,9-11], in which central factors are also involved in the mechanism of glucocorticoid- and/or stress-induced suppression of the HPO axis [6,12,13].

Nevertheless, the research on the direct effects of glucocorticoids on ovarian reproductive functions has been very limited [14-17]. In the present study, primary granulosa cells obtained from rat ovaries were used to clarify the direct effects of glucocorticoids on ovarian steroidogenesis integrated by follicle-stimulating hormone (FSH) and various growth factors expressed in the ovary. Mutual interactions among gonadotropins and the growth factors including bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs) and activins/inhibins are critical for normal growth and maturation of ovarian follicles [18,19]. Among these factors, the ovarian BMPs play regulatory roles on the

activity of FSH receptor (FSHR) expressed on granulosa cells, leading to inhibition of progesterone biosynthesis and luteinizing process [20-22]. The present study elucidated specific activity of glucocorticoids in the enhancement of progesterone production by granulosa cells and the functional interaction with the ovarian BMP system.

Materials and Methods

Reagents and supplies

Dexamethasone (Dex), diethylstilbestrol (DES), ovine FSH, 4-androstene-3,17-dione (androstenedione: a substrate for aromatase) and 3-isobutyl-1-methylxanthine (IBMX: a phosphodiesterase inhibitor) were purchased from Sigma-Aldrich Co. Ltd. (St. Louis, MO). Recombinant protein of human BMP-2, -4, -9 and -15 was purchased from R&D Systems Inc. (Minneapolis, MN).

Primary culture of rat granulosa cells

DES (10 mg) packed in silastic tubes was implanted subcutaneously in the back of 22-day-old female Sprague-Dawley rats (Charles River, Wilmington, MA). After three days of exposure to DES, the ovaries were resected and punctured

with a 27-gauge needle. Granulosa cells were separated from oocytes by passing the cell suspension in Medium 199 (Thermo Fisher Scientific, Waltham, MA) through nylon meshes of different pore sizes (100 and 40 μm) (BD Falcon, Bedford, MA), as we previously reported [23-25]. The granulosa cells were cultured in McCoy's 5A (Thermo Fisher Scientific) supplemented with penicillin-streptomycin without serum. The animal protocol was approved by Okayama University Institutional Animal Care and Use Committee (OKU-2018704).

Measurements of ovarian steroids and cAMP

Granulosa cells were seeded in 96-well plates with McCoy's 5A (1×10^5 viable cells in 200 μl) and androstenedione (100 nM). The cells were cultured for 48 h after adding indicated concentrations of Dex to the medium either alone or with FSH (10 ng/ml). The levels of ovarian steroids in the medium were measured using estradiol and progesterone enzyme-linked immunosorbent assay (ELISA) kits (Cayman Chemical Co., Ann Arbor, MI). The levels of steroids were undetectable in the cell-free medium (progesterone < 10 pg/ml and estradiol < 15 pg/ml). Cellular synthesis of cAMP was evaluated in granulosa cells (1×10^5 viable cells in 200 μl) cultured with IBMX (0.1 mM) for 48 h in 96-well plates.

The concentrations of extracellular cAMP in the medium were measured by a cAMP ELISA kit after acetylation, and the assay sensitivity was 0.039 nM (Enzo Life Sciences, Inc., Farmingdale, NY).

Quantitative reverse transcription-PCR of extracted RNA

Granulosa cells (5×10^5 viable cells in 1 ml) were cultured in 12-well plates with serum-free McCoy's 5A and were treated with FSH (10 ng/ml) and BMP ligands (100 ng/ml) either alone or with Dex (100 nM). After the treatments for 24 to 48 h, total RNA was extracted using TRI Reagent® (Cosmo Bio Co. Ltd., Tokyo) and was quantified using a NanoDropTM One spectrophotometer (Thermo Fisher Scientific). The primers for genes related to steroidogenesis including *Star* (also indicated as StAR), *Cyp11a* (P450scc), *Hsd3b* (3βHSD), and *Cyp19* (P450arom), for genes related to the BMP receptor and the signaling including *Acvr1* (ALK-2), *Bmpr1a* (ALK-3), *Bmpr1b* (ALK-6), *Bmpr2* (BMPRII), *Acvr2a* (ActRIIA), *Acvr2b* (ActRIIB), *Smad6*, *Smad7*, and *Id1* (inhibitor of DNA binding 1: the BMP target gene), for *Gr* (glucocorticoid receptor), and for a housekeeping gene, *Rpl19* (RPL19) were chosen on the basis of previous reports [23-27]. The primer pairs were selected from different exons of the corresponding genes to distinguish the

target PCR products from contaminants in the chromosomal DNA. The primer pairs for *Hsd20a* (20αHSD) were selected from 776-795 and 1027-1046 of gene accession# AB028066. The RNA (1 μg) was reverse-transcribed using ReverTra Ace® (TOYOBO Co., Ltd. Osaka) with random primers and deoxynucleotide triphosphate. Quantitative PCR was performed using the LightCycler® 96 system (Roche Diagnostic Co., Tokyo) under the appropriate annealing conditions and amplification efficiency. The mRNA expression levels were quantified relatively using the comparative threshold cycle (Ct) method and were expressed as $2^{-(\Delta\Delta Ct)}$ using *Rpl19* as the internal control.

Western blotting

Granulosa cells (2.5×10^5 viable cells in 500 μl) were cultured in 24-well plate in serum-free McCoy's 5A. After culturing for 24 h either alone or with Dex (100 nM), the cells were treated with BMP-15 (100 ng/ml) for 60 min. The treated cells were solubilized in 100 μl of RIPA lysis buffer (Upstate Biotechnology, Lake Placid, NY) supplemented with 4% β-mercaptoethanol and 2% SDS. The cell lysates were subjected to SDS-PAGE/immunoblotting analysis with antibodies against phopho-Smad1/5/9 (pSmad1/5/9), total-Smad1 (tSmad1; Cell Signaling

Technology, Inc., Beverly, MA) and actin (Sigma-Aldrich Co. Ltd.). Each band was analyzed using the C-DiGit® Blot Scanner System (LI-COR Biosciences, Lincoln, NE). The target signal intensities were expressed as a relative value to the corresponding actin intensities.

Statistics

The results are presented as means ± SEM of at least three separate experiments with triplicated samples. Statistical analysis was performed using ANOVA followed by Fisher's protected least significant difference (PLSD) test and the unpaired t-test (StatView 5.0 software, Abacus Concepts, Inc., Berkeley, CA). A significant difference was defined as P values < 0.05.

Results

We first examined the effects of Dex treatment on ovarian steroidogenesis by rat primary granulosa cells. After treatment with Dex (1 to 300 nM) for 48 h in a serum-free condition, the changes in estradiol and progesterone production induced by FSH treatments were examined. As shown in **Fig. 1A**, it was revealed that Dex treatment reduced estradiol production

1 induced by FSH (10 ng/ml) but increased progesterone production induced by
2 FSH by granulosa cells in a concentration-dependent manner (**Fig. 1A**).
3 To try to determine the mechanism by which Dex regulates ovarian
4 steroidogenesis, the mRNA expression levels of steroidogenetic protein and
5 enzymes were evaluated. Total RNAs of granulosa cells treated with FSH (10
6 ng/ml) or Dex (100 nM) for 48 h were extracted and mRNA levels of
7 steroidogenetic factors and enzymes were quantified by real-time RT-PCR. In
8 accordance with the effects of Dex on estradiol synthesis, Dex (100 nM)
9 treatment suppressed P450arom mRNA expression (**Fig. 2A**). The cAMP
10 synthesis stimulated by FSH (10 ng/ml) was also suppressed by Dex (100 nM)
11 treatment for 48-h culture of granulosa cells (**Fig. 2B**). As shown in **Fig. 2C**,
12 Dex treatment significantly enhanced the expression of mRNAs encoding the
13 enzymes for progesterone synthesis including P450scc and 3βHSD, but not StAR,
14 induced by FSH (10 ng/ml). Basal levels of mRNAs of P450scc and 3βHSD
15 were also increased by treatment with Dex (100 nM) for 48 h (**Fig. 2B**), although
16 the mRNA levels of StAR and 20αHSD, which alters progesterone to an inactive
17 form, were not affected by the Dex treatment. The enzyme 20αHSD
18 predominantly converts progesterone to its biologically inactive form, 20α-

hydroxyprogesterone, and has an important role in the termination of pregnancy and the initiation of parturition [28,29].

Next, the effects of Dex on BMP receptor signaling by granulosa cells were evaluated. Total RNAs of granulosa cells treated with BMPs (100 ng/ml) in combination with Dex (100 nM) for 48 h were extracted and mRNA levels of steroidogenetic factors and enzymes were quantified by quantitative PCR. Of note, Dex treatment significantly upregulated transcription of the BMP-target gene Id-1 induced by BMP-15 (100 ng/ml) compared with that induced by other BMP ligands including BMP-2, -4 and -9 (**Fig. 3A**). Furthermore, Western blot analysis using cell lysates showed that treatments with both BMP-15 and Dex effectively upregulated Smad1/5/9 phosphorylation by granulosa cells (**Fig. 3B**). It was also found that Dex treatment increased the expression level of the BMP type-I receptor ALK-6 among the type-I and -II receptors for BMP-15 (**Fig. 3C**). Inhibitory Smad6/7 expression was not affected by Dex treatment. On the other hand, BMP-15 treatment upregulated GR expression in granulosa cells (**Fig. 3D**). Thus, it was revealed that glucocorticoids elicit differential effects on ovarian steroidogenesis, in which GR and BMP-15 actions are mutually enhanced in granulosa cells.

Discussion

In the present study, it was revealed that Dex treatment decreased estradiol production but increased progesterone production induced by FSH by granulosa cells *in vitro* (**Fig. 4**). Dex treatment also suppressed P450arom mRNA expression and cAMP synthesis induced by FSH, whereas Dex enhanced basal as well as FSH-induced levels of mRNAs encoding the enzymes for progesterone synthesis including P450scc and 3βHSD. Importantly, Dex treatment was found to upregulate BMP-Smad1/5/9 signaling in the presence of BMP-15 among the key ovarian BMP ligands via upregulating ALK-6, which is the main type-I receptor for BMP-15, while BMP-15 upregulated GR expression in granulosa cells. Namely, glucocorticoids can elicit differential effects on ovarian steroidogenesis, in which GR and BMP-15 activities seem to be mutually enhanced in granulosa cells (**Fig. 4**).

In the ovarian follicles, the BMP system including BMP ligands and the receptors has been known to be expressed in a cell-specific manner [20]. In mammals, the ovarian BMP system has been shown to play crucial roles in the maintenance of female fertility [19-22]. In addition to the ligand-dependent

actions of BMPs in the process of normal folliculogenesis, a common key function of BMPs has been recognized to regulate the FSHR sensitivity expressed on granulosa cells, [30-32]. For instance, the expression of BMP-6 was predominantly enhanced in granulosa cells derived from ovarian tissues of PCOS patients compared with normal patients [33-35], implying that upregulation of BMPs are involved in the disruption of follicular development in the ovaries of PCOS. In the present study, the expression level of the BMP type-I receptor ALK-6 was revealed to be upregulated by glucocorticoid treatment, implying a functional interaction between GR and the BMP system in granulosa cells.

Heush et al. [14] reported the effects of glucocorticoids on steroidogenesis of ovarian granulosa cells. Treatments with cortisol and dexamethasone suppressed the increase in P450arom activity induced by FSH in rat granulosa cell culture. In the same culture condition, progesterone production was stimulated to a maximum of ~1.7 fold of the control level. These differential effects of glucocorticoids on estrogen production and progesterone production indicate that glucocorticoids elicit selective inhibition of P450arom activity induced by FSH via a mechanism other than interfering with the aromatization reaction in granulosa cells.

In this regard, Adashi et al. [15] also reported direct effects of glucocorticoids on basal and FSH-stimulated production of progesterone by ovarian granulosa cells from immature, hypophysectomized, estrogen-treated rats. In that study, the stimulatory effects of the corticoids tested were correlated with glucocorticoid rather than mineralocorticoid potencies. As for the underlying mechanism, treatment with glucocorticoids led to a significant decrease in the activity of 20αHSD detected by the conversion rate of progesterone, leading to an increase in progesterone production [15]. However, the results were not reproduced by our experiments, which showed that 20αHSD mRNA expression was not altered by Dex treatment in granulosa cells.

In addition to glucocorticoid activity in granulosa cells, we found in our earlier study that aldosterone (Aldo) treatment enhanced FSH-induced progesterone, but not estradiol, production [36]. Of interest, it was found that Aldo treatment suppressed BMP-6-induced Smad1/5/9 signaling with increased expression of inhibitory Smad6. On the other hand, mineralocorticoid receptor (MR) expression in granulosa cells was downregulated by BMP-6, showing the functional interaction as a negative feedback between BMP and MR signaling. Given that the ovarian BMP system acts as suppressor for progesterone

biosynthesis as a luteinizing inhibitor [20-22], Aldo plays an inhibitory role in the endogenous BMPs, leading to the progesterone accumulation in the growing follicles.

Increased androgen action and its receptor activity in various stages of developing follicles have been shown to be involved in the pathogenesis of PCOS [37]. We previously reported that androgen in combination with insulin-like growth factor-I enhanced FSH-induced progesterone synthesis by rat granulosa cells [23]. The impact of androgen action that enhanced progesterone production was similar to the effects of Aldo, both of which are functionally linked to the counteraction of endogenous BMPs in granulosa cells, leading to the enhancement of progesterone biosynthesis. However, the effects of Dex on BMP activity, which was different from that of androgen or Aldo, resulted in the upregulation of BMP-15 signaling. This finding implies that the upregulation of BMP-receptor signaling may reflect a negative feedback function to suppress progesterone production directly enhanced by Dex treatment by granulosa cells.

In a clinical setting, glucocorticoids such as prednisone and dexamethasone have been used to induce ovulation [2]. In PCOS patients with a high adrenal androgen level, low-dose dexamethasone can be used to attain a

higher ovulation rate and higher cumulative pregnancy rate. Such effects of glucocorticoids on successful ovulation and/or pregnancy may be involved in the activity of glucocorticoids on the enhancement of progesterone as well as BMP signaling in granulosa cells as shown in the present study.

Collectively, the results indicated that glucocorticoids elicit differential effects on ovarian steroidogenesis of estradiol and progesterone, in which GR and BMP-15 actions are mutually enhanced in granulosa cells. Given that BMP-15 acts as an inhibitor for progesterone production by suppressing FSH-receptor actions [38,39], the results suggested that glucocorticoids are functionally linked to the enhancement of endogenous BMP-15, leading to negative feedback toward the progesterone overproduction induced by FSH and Dex in granulosa cells (**Fig. 4**). Hence, it was revealed that glucocorticoids elicit differential effects on ovarian steroidogenesis, in which GR and BMP-15 actions are mutually enhanced in granulosa cells.

Acknowledgements

This work was supported in part by Grants-in-Aid for Scientific Research (18K08479 and 21K08556).

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1 **Figure Legends:**

2 **Fig. 1. Effects of glucocorticoid on FSH-induced steroidogenesis by rat**
3 **granulosa cells.** Granulosa cells were cultured with indicated concentrations
4 of dexamethasone (Dex) either alone or in combination with FSH in a serum-free
5 condition for 48 h. The concentrations of **A**) estradiol and **B**) progesterone in
6 the medium were evaluated by ELISA and expressed as fold changes. Results
7 in all panels are shown as means ± SEM of data from at least three individual
8 experiments with triplicated samples. The results were analyzed by ANOVA with
9 Fisher's PLSD test.

10

11 **Fig. 2. Effects of glucocorticoid on steroidogenetic enzyme expression by**
12 **rat granulosa cells.** **A**, **C**) Cellular RNA was extracted from granulosa cells
13 treated with FSH and Dex for 48 h. Cyp19, *Star*, *Cyp11a*, and *Hsd3b* mRNA
14 levels were determined by real-time PCR. The target gene mRNA levels were
15 standardized by *Rpl19* levels and expressed as fold changes. **B**) Cells were
16 cultured with Dex either alone or in combination with FSH in a serum-free medium
17 containing IBMX for 48 h. The cAMP levels in the medium were examined by
18 ELISA. Results in all panels are shown as means ± SEM of data from at least

three individual experiments with triplicated samples. The results were analyzed by ANOVA with Fisher's PLSD test and the unpaired *t*-test. Values with different superscript letters show significant difference at $P < 0.05$; and $^{*}P < 0.05$ vs. control group.

Fig. 3. Effects of glucocorticoid activity on BMP signaling in rat granulosa cells. **A**, **C**) Cellular RNA was extracted from granulosa cells treated with BMP-2, - 4, -9 and -15 in the presence or absence of Dex for 24 h, and *Id1* mRNA levels were determined by real-time PCR. The target gene mRNA levels were standardized by *Rpl19* levels and expressed as fold changes. **B**) Cells were cultured in a serum-free condition in the presence or absence of Dex for 48 h. The cells were lysed and subjected to Western blot analysis using anti-Smad1/5/9, tSmad1 and anti-actin antibodies. The signal intensities of pSmad1/5/9 and Smad6 were standardized by actin signal intensities in each sample and expressed as fold changes. **C**, **D**) RNA was extracted from granulosa cells treated with Dex or BMP-15 for 48 h and mRNA levels of BMP receptors, inhibitory Smads and Gr were determined by real-time PCR. Results in all panels are shown as means ± SEM of data from at least three individual

experiments with triplicated samples. The results were analyzed by ANOVA with Fisher's PLSD test and the unpaired *t*-test. *$P < 0.05$ vs. control group.

Fig. 4. Mechanism by which glucocorticoid regulates steroidogenesis via BMP-Smad signaling in granulosa cells. Dex decreased estradiol production but increased progesterone production induced by FSH by granulosa cells. Dex also suppressed P450arom mRNA expression and cAMP synthesis induced by FSH, whereas Dex enhanced basal as well as FSH-induced levels of mRNAs encoding the enzymes for progesterone synthesis including P450scc and 3βHSD. Dex upregulated BMP-15/Smad1/5/9 signaling via upregulating the BMP type-I receptor, while BMP-15 upregulated GR expression. GR and BMP-15 actions were mutually enhanced in granulosa cells. AC, adenylate cyclase; BMPRs, BMP receptors; and FSHR, FSH receptor.