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Involvement of bone morphogenetic protein activity in somatostatin actions on ovarian steroidogenesis

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ABSTRACT

Somatostatin is expressed in the hypothalamus, pancreas and gastrointestinal tracts and it inhibits the secretion of various hormones in vivo. In the rodent ovary, somatostatin receptor (SSTR) subtypes 2 and 5 are expressed in granulosa cells and oocytes. Somatostatin analogs have been clinically used for treatment of endocrine tumors. For this purpose, relatively high-dose or long-term treatments of somatostatin analogs are necessary; however, the direct and continuous impact of somatostatin analogs on gonadal functions has yet to be elucidated. In the present study, we investigated the effects of somatostatin analogs (octreotide and pasireotide) on ovarian steroidogenesis by rat primary granulosa cell culture. The expression levels of SSTR2 and SSTR5 in granulosa cells were upregulated by FSH treatment. Treatment with somatostatin analogs decreased FSH-induced estradiol production with reduction in aromatase mRNA expression, while the treatment also suppressed FSH-induced progesterone production with reduction of mRNAs levels of StAR, P450scc and 3βHSD2 in granulosa cells. This trend was also observed in a granulosa/oocyte co-culture condition. The effect of pasireotide was more potent than that of octreotide. FSH-induced synthesis of steroids and cAMP was also suppressed by somatostatin analog treatment. Notably, pretreatment with a BMP-binding protein, noggin reversed the suppressive effects of somatostatin analogs on progesterone and cAMP production, suggesting that the endogenous BMP system is functionally involved in the SSTR effects in granulosa cells. Treatment with BMP-2, -4, -6 and -7 decreased the mRNA expression of inhibitory Smads6 and 7, leading to enhancement of BMP actions detected by Id-1 transcription in granulosa cells. Collectively, the results revealed that SSTR activation modulates ovarian steroidogenesis by upregulating endogenous BMP activity in growing follicles.

1. Introduction

Somatostatin is a 14-amino-acid polypeptide with a short half-life that is expressed in the hypothalamus, pancreas and gastrointestinal tract. It inhibits the secretion of various hormones including growth hormone (GH) and luteinizing hormone (LH) [1] and the release of pancreatic insulin [2]. In the rodent ovary, somatostatin receptor (SSTR) subtypes 2 and 5 are mainly expressed in granulosa cells and oocytes [3,4]. Several kinds of somatostatin analogs have been clinically used for the treatments of functioning pituitary adenomas and various neuroendocrine tumors at much higher concentrations compared with endogenous levels of somatostatin.

Earlier studies in various species have shown that somatostatin and its receptors are present in the ovary. As for the endogenous somatostatin, McIntyre et al. demonstrated the protein expression of somatostatin in the rat ovary [5]. The expression of immune-reactive somatostatin was detected in granulosa cells. Somatostatin molecules in the conditioned medium of granulosa cell culture contained equal levels of peaks of somatostatin-14 and -28, despite theprepro-somatostatin mRNA expression was not detectable in the ovary [5]. Therefore, it is presumable that the

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; FSK, forskolin; 3βHSD, 3β-hydroxysteroid dehydrogenase; P450arom, P450 aromatase; P450scc, P450 steroid side-chain cleavage enzyme; PCOS, polycystic ovary syndrome; SSTR, somatostatin receptor; StAR, steroidogenic acute regulatory protein; TGF-β, transforming growth factor-β.

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ovarian expression of somatostatin occurs only within a restricted period and/or that somatostatin produced at extra-gonadal sites may be just stored or concentrated in granulosa cells in the ovary.

Furthermore, Nestorovic et al. [6] reported that somatostatin administered to peripubertal mice not only decreased circulating follicle-stimulating hormone (FSH) and LH levels, but also blocked the initial gonadotropin-independent process of follicle growth in vivo, suggesting that somatostatin might directly act on follicular component cells in the ovary. A recent study by Gougeon et al. using a somatostatin receptor antagonist, BIM23627, also showed that somatostatin is involved in mouse folliculogenesis by reducing recruitment of resting follicles [4].

The effects of somatostatin are mediated through G protein-coupled SSTR1–5, and the receptors can be activated by various somatostatin analogs having different affinities for the five receptors. Octreotide is an SSTR2-prefering agonist, and pasireotide (also called SOM230) is a multi-receptor agonist that binds to SSTR1–3 and 5 with high affinity, having a 40-fold higher and prolonged affinity to SSTR5 compared with that of octreotide [7,8]. However, the direct effects of octreotide and pasireotideon the regulatory mechanism of gonadal functions have yet to be elucidated.

Ovarian follicle growth and maturation occur via complex interactions between pituitary gonadotropins and numerous autocrine/paracrine growth factors produced within the ovary. Recent studies have established the concept that members of the transforming growth factor (TGF)-β superfamily, including bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs) and activins/inhibins, play key roles as autocrine/paracrine factors in female fertility in mammals [9]. The regulation of FSH responsiveness in granulosa cells is critical for the establishment of dominant follicles and subsequent ovulation in mammals. FSH receptor (FSHR) signaling in granulosa cells is required for follicular selection and dominant follicle formation. The FSH-regulated follicle selection and dominant follicle formation are precisely modulated by autocrine/paracrine factors within the follicles [10]. BMPs play a key role in female fertility by regulating steroidogenesis and mitosis in granulosa cells. Furthermore, BMP ligands suppress FSH-induced progesterone production as luteinizing inhibitors [9]. The major regulatory cue by BMPs in folliculogenesis is control of FSH receptoractivity in granulosa cells.

Given that somatostatin is an inhibitory regulator of a variety of endocrine systems, including cAMP synthesis in various organs, it is possible that somatostatin and a luteinizing inhibitor, namely BMP family member, have a functional link regarding steroidogenesis induced by FSH in granulosa cells. In this study, we investigated the interaction of BMP system and the somatostatin effects at pharmacologic doses in folliculogenesis by focusing on ovarian steroidogenesis.

2. Materials and methods

2.1. Reagents and supplies

Female Sprague-Dawley (SD) rats were purchased from Charles-River (Wilmington, MA). Medium 199, McCoy's 5A medium and HEPES buffer solution were purchased from Invitrogen Corp. (Carlsbad, CA). Diethylstilbestrol (DES), ovine pituitary FSH, forskolin (FSK), 4-androstene-3,17-dione, 3-isobutyl-1-methylxanthine (IBMX), bovine serum albumin, and penicillin–streptomycin solution were from Sigma–Aldrich Co. Ltd. (St. Louis, MO). Pasireotide (SOM230) and octreotide acetate were kindly provided by Novartis International Pharmaceutical Ltd. (Basel, Switzerland). Recombinant human BMP-2, -4, -6 and -7 and mouse noggin were purchased from R&D Systems Inc. (Minneapolis, MN).

2.2. Primary culture of granulosa cells and co-culture with oocytes

Silastic capsules containing 10 mg of DES were implanted in 22-day-old female SD rats to increase the number of granulosa cells. After 4 days of DES exposure, ovarian follicles were punctured with a 28-gauge needle, and the isolated mixture of granulosa cells and oocytes was cultured in serum-free McCoy's 5A medium. For indicated experiments, granulosa cells were separated from oocytes by filtering the oocyte/granulosa cell suspension through an additional 40-μm nylon mesh (BD Falcon) that allowed granulosa cells but not oocytes to pass through [11,12]. The animal protocols were approved by Okayama University Institutional Animal Care and Use Committee.

2.3. Measurements of estradiol, progesterone and cAMP levels

Rat granulosa cells (1×10^5 viable cells in 200 μl) with or without oocytes (100 oocytes/ml) were cultured in 96-well plates with serum-free medium containing 100 nM of and rostenedione. FSH (30 ng/ml) or forskolin (10 μM) was added to the culture medium either alone or in combination with indicated concentrations of octreotide, pasireotide, BMP-2, -4, -6, -7 and noggin. After 48-h culture, the culture media were collected and stored at −80 °C until assay. The levels of estradiol and progesterone in the media were determined by a chemiluminescent immunoassay (CLIA) [13]. To assess cellular cAMP synthesis, rat granulosa cells (1×10^5 viable cells in 200 μl) were cultured in serum-free medium containing 0.1 mM of IBMX. After 48-h culture with indicated treatments, the extracellular contents of cAMP were determined by an enzyme immunoassay (EIA) [13].

2.4. Cellular RNA extraction, RT and quantitative real-time PCR

Rat granulosa cells (5×10^5 viable cells in 1 ml) and collected oocytes (~5000/ml) were cultured in 12-well plates with serum-free McCoy's 5A medium. FSH (30 ng/ml) was added to the culture medium either alone or in combination with indicated concentrations of somatostatin analogs, BMPs and noggin. After 48-h culture, the medium was removed and total cellular RNA was extracted using TRIzol (Invitrogen Corp.). Primer pairs for P450arom, P450scc, steroidogenic acute regulatory protein (StAR), 3β-hydroxysteroid dehydrogenase type 2 (3βHSD2), ALK-2, -3 and -6, ActRII, BMPRII, Smad1-8, SSTR1-5 and ribosomal protein L19 (RPL19) were selected as reported previously [14–17]. The extracted RNA (1 μg) was subjected to an RT reaction. For the quantification of each mRNA expression, real-time PCR was performed using LightCycler-FastStart DNA master SYBR Green I system and LightCycler Nano System (Roche Diagnostic Co., Tokyo, Japan) following the manufacturer's protocol. Accumulated levels of fluorescence for each product were analyzed by the second derivative method (Roche Diagnostic Co.) and then the expression levels of target genes and a house-keeping gene, RPL19, were quantified [13].

2.5. Statistical analysis

All results are shown as means ± SEM of data from at least three separate experiments, each performed with triplicate samples. The data were subjected to ANOVA or the unpaired *t*-test (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.