Journal of Steroid Biochemistry and Molecular Biology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Interaction between orexin A and bone morphogenetic protein system on progesterone biosynthesis by rat granulosa cells

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ARTICLE INFO

Keywords:
Bone morphogenetic protein (BMP)
Follicle-stimulating hormone (FSH)
Granulosa cells
Noggin
Orexin
Steroidogenesis

ABSTRACT

The involvement of orexins in reproductive function has been gradually uncovered. However, the functional role of orexins in ovarian steroidogenesis remains unclear. In the present study, we investigated the effects of orexin A on ovarian steroidogenesis by using rat primary granulosa cells that express both OX1 and OX2 receptors for orexins. Treatment with orexin A enhanced progesterone, but not estradiol, biosynthesis induced by FSH, whereas it did not affect basal levels of progesterone or estradiol. In accordance with the effects on steroidogenesis, orexin A increased the mRNA levels of progesterogenic enzymes, including StAR, P450scc and 3βHSD, but not P450arom, and cellular cAMP synthesis induced by FSH. Under the condition of blockage of endogenous BMP actions by noggin or BMP-signaling inhibitors, orexin A failed to increase levels of progesterone synthesis induced by FSH treatment, suggesting that endogenous BMP activity in granulosa cells might be involved in the enhancement of progesterone synthesis by orexin A. Treatment with orexin A impaired Smad1/5/9 activation as well as Id-1 mRNA expression stimulated by BMP-6 and BMP-7, the latter of which was reversed by treatment with an OX1 antagonist. It was also found that orexin A suppressed the mRNA expression of both type-I and -II receptors for BMPs and increased that of inhibitory Smad6 and Smad7 in granulosa cells. On the other hand, treatments with BMP-6 and -7 suppressed the expression of OX1 and OX2. Collectively, the results indicated that orexin A enhances FSH-induced progesterone production, at least in part, by down-regulating BMP signaling in granulosa cells. Thus, a new role of orexin A in facilitating progesterone synthesis and functional interaction between the orexin and BMP systems in granulosa cells were revealed.

1. Introduction

Orexins A and B are neuropeptides that are mainly synthesized in the hypothalamus in the process of proteolytic cleavage from the common precursor prepro-orexin [1,2]. Orexins play key roles in the control of sleep-wakefulness, energy balance and food intake. The actions of orexins are mediated *via* two orexin receptors, OX1 and OX2: OX1 is selective for orexin A and OX2 binds both orexin A and orexin B [3]. It has also been shown that orexins and their receptors are expressed in various peripheral tissues outside the central nervous system [4]. Recently, the existence of an interrelationship between the orexinergic and reproductive systems, including the actions of orexins on hypothalamic gonadotropin-releasing hormone (GnRH) neurons and pituitary gonadotropes, has been recognized [5,6].

As for the action of orexins in the hypothalamic-pituitary-ovarian (HPO) axis, Silveyra et al. [7] reported that orexin receptors were expressed in the brain and pituitary of female cycling rats and that the expression levels of OX1, OX2 and prepro-orexin were increased in the hypothalamus and pituitary during the proestrus evening. Nitkiewicz et al. [8] also confirmed the expression of OX1 and OX2 in porcine

Abbreviations: AC, adenylate cyclase; ALK, activin receptor-like kinase; ActRII, activin type-II receptor; BMP, bone morphogenetic protein; BMPRII, BMP type-II receptor; DOR, dorsomorphin; FSH, follicle-stimulating hormone; FSHR, FSH receptor; GDF, growth and differentiation factor; GnRH, gonadotropin-releasing hormone; HPO, hypothalamic-pituitary-ovarian; 3βHSD, 3β-hydroxysteroid dehydrogenase; LDN, LDN193189; ORX, orexin A; OX1 and OX2, orexin receptor type 1 and type 2; P450arom, P450 aromatase; P450scc, P450 steroid side-chain cleavage enzyme; StAR, steroidogenic acute regulatory protein

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https://doi.org/10.1016/j.jsbmb.2018.03.004
Received 12 November 2017; Received in revised form 27 February 2018; Accepted 11 March 2018

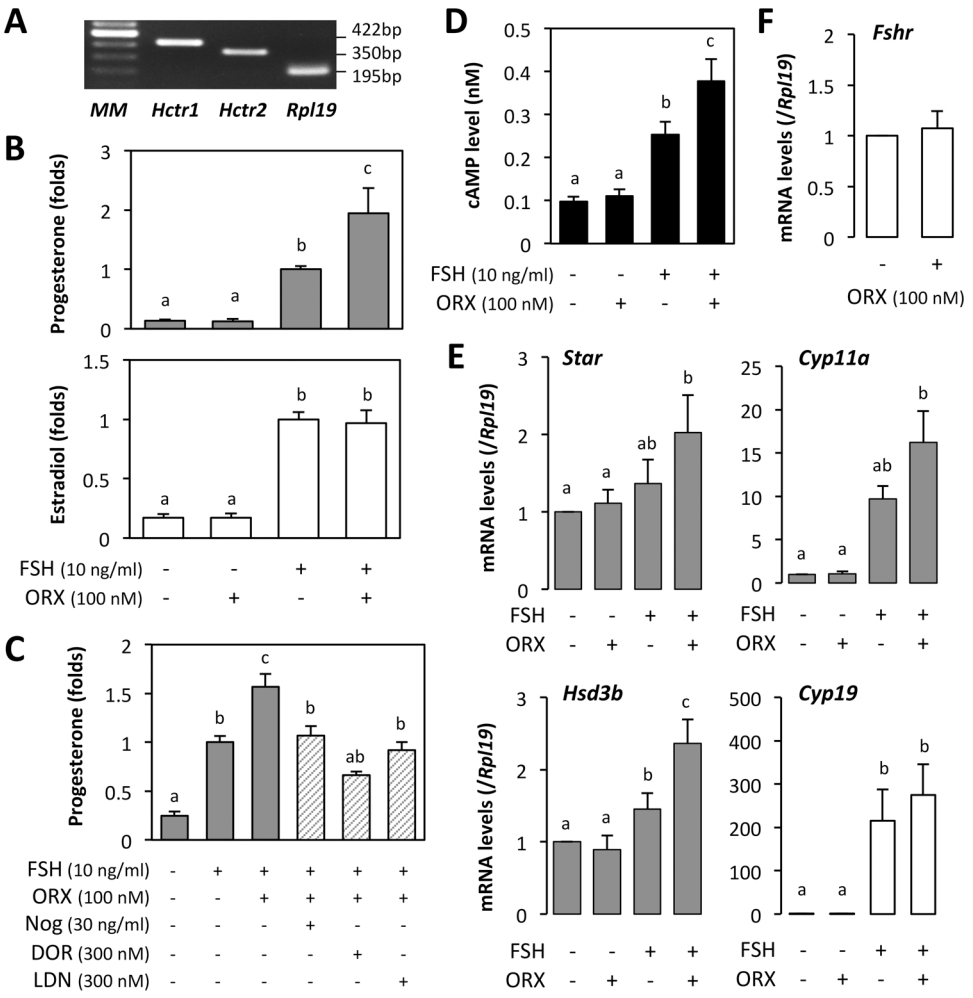

Fig. 1. Expression of orexin receptors and effects oforexin A on FSH-induced steroidogenesis by rat granulosa cells. A) Total cellular RNA was extracted from granulosa cells, and the expression of *Hctr1* (422 bp), *Hctr2* (350 bp) and *Rpl19* (195 bp) was examined by RT-PCR. B) Granulosa cells were cultured in a serum-free condition with FSH either alone or in combination with orexin A (ORX). After 48-h culture, the levels of progesterone and estradiol in the medium were determined by CLIA, and the levels of progesterone and estradiol were expressed as fold changes. C) Granulosa cells were cultured in the presence of noggin (Nog), dorsomorphin (DOR) and LDN193189 (LDN) in a serum-free condition with FSH either alone or in combination with ORX. After 48-h culture, the levels of progesterone in the medium were determined by CLIA, and the levels of progesterone were expressed as fold changes. D) Granulosa cells were cultured in a serum-free medium containing IBMX with FSH either alone or in combination with ORX. After 48-h culture, the levels of cAMP in the medium were determined by EIA. E, F) Total cellular RNA was extracted from granulosa cells treated with FSH and ORX for 48 h in a serum-free condition, and *Star*, *Cyp11a*, *HSD3b*, *Cyp19* and *Fshr* mRNA levels were determined by quantitative PCR. The expression levels of target gene mRNA were standardized by *Rpl19* level and expressed as fold changes. Results in all panels are shown as means ± SEM of data from at least three separate experiments, each performed with triplicate samples. The results were analyzed by ANOVA. Values with different superscript letters are significantly different at $P < 0.05$. MM indicates molecular weight marker.

ovaries at the gene and protein levels and showed that the levels of OX1 and OX2 expression changed according to the cycle. The cycle-dependent expression of orexin receptors suggested physiological significance of the ovarian orexin system. The finding that orexin ligands exist in all follicular stages of adult ovaries of other species such as cats and dogs indicated that the ovary might be a major potential site of orexinergic activity [9].

Regarding ovarian steroidogensis in relation to orexin, Cataldi et al. [10] found that the expression levels of OX1 and OX2 were increased and the production of progesterone was reduced by treatment of luteal cells from superovulated rat ovaries with orexins. Inhibitory effects of orexin on estradiol synthesis induced by follicle-stimulating hormone (FSH) in porcine granulosa cells also suggested a regulatory role of orexins in reproductive functions through modulation of ovarian steroidogenesis [11].

On the other hand, recent studies have demonstrated that ovarian growth factors play critical roles in female fertility in mammals in an autocrine/paracrine manner [12]. Complex interactions between gonadotropins and ovarian autocrine/paracrine factors including activins/inhibins, bone morphogenetic proteins (BMPs) and growth differentiation factors (GDFs) are critical for follicle growth and maturation. BMP system molecules consisting of the units of BMP ligands and receptors are cell-specifically expressed in ovarian follicles. The ovarian BMP system regulates FSH receptor activity in granulosa cells, leading to a critical control of ovarian folliculogenesis in an autocrine/paracrine manner [12–14]

In the present study, we used a primary culture of rat granulosa cells to investigate the functional roles of orexins, particularly in steroidogenesis induced by FSH with focus on the ovarian BMP system. The results of experimental studies suggested the involvement of orexins at various points of the reproductive axis; however, the impact of orexins on steroidogenetic cascades has yet to be determined. The present study uncovered a novel activity of orexins in progesterone biosynthesis and its functional interaction with the ovarian BMP system in granulosa cells.

2. Materials and methods

2.1. Experimental reagents and supplies

Culture media including HEPES buffer solution, McCoy's 5A and Medium 199 were purchased from Invitrogen Corp. (Carlsbad, CA). Hormones and chemicals including 4-androstene-3,17-dione, diethylstilbestrol (DES), 3-isobutyl-1-methylxanthine (IBMX), ovine pituitary FSH, and penicillin-streptomycin solution were purchased from Sigma-Aldrich Co. Ltd. (St. Louis, MO). Recombinant proteins including human BMP-6 and BMP-7 and mouse Noggin were obtained from R&D Systems Inc. (Minneapolis, MN), and human Orexin A was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). BMP-receptor signaling inhibitors, LDN193189 and dorsomorphin, were from Stemgent (San Diego, CA) and Calbiochem (San Diego, CA), respectively. A selective non-peptide OX1 antagonist, SB408124 [15], was purchased from Tocris Bioscience (Bristol, UK).