Domestic Animal Endocrinology 44 (2013) 115–120

Contents lists available at SciVerse ScienceDirect

Domestic Animal Endocrinology

journal homepage: www.domesticanimalendo.com

Stage-specific expression and effect of bone morphogenetic protein 2 on bovine granulosa cell estradiol production: regulation by cocaine and amphetamine regulated transcript

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ARTICLE INFO

Article history:
Received 18 July 2012
Received in revised form 10 October 2012
Accepted 11 October 2012

Keywords:
Ovary
Bovine
Granulosa cell
BMP2
CART

ABSTRACT

Members of the bone morphogenetic protein (BMP) family regulate follicular development and granulosa cell function. However, changes in expression of BMP2 and its receptors during follicular waves in cattle and ability of BMP2 to modulate bovine granulosa cell estradiol production are not well understood. The objectives of this study were to determine temporal regulation of mRNA for *BMP2* and its type I and II receptors (*BMPR1A* and *BMPR2*) in bovine follicles collected at specific stages of a follicular wave (predeviation, early dominance, mid dominance, preovulatory), ability of BMP2 to modulate bovine granulosa cell steroidogenesis, and whether effects of BMP2 on granulosa cell estradiol production are influenced by cotreatment with cocaine- and amphetamine-regulated transcript (CART), an intrafollicular regulatory peptide shown to inhibit estradiol production in response to other trophic hormones (FSH and IGF1). Relative abundance of mRNAs for *Bmp2* and *Bmpr2* was elevated at the mid dominance stage relative to earlier stages of the follicular wave and further increased at the preovulatory stage. Abundance of mRNA for *Bmpr1a* was lowest at early dominance stage and highest at preovulatory stage relative to other stages of the follicular wave examined. Treatment of bovine granulosa cells in vitro with BMP2 increased estradiol but decreased progesterone concentrations. Co-incubation with CART reduced the BMP2-stimulated increase in granulosa cell estradiol production. Results suggest that BMP2 may play a regulatory role in development of bovine follicles to the preovulatory stage and that CART can inhibit granulosa cell estradiol production in response to multiple hormones/growth factors, including BMP2.

1. Introduction

Antral follicle development occurs in a characteristic wave-like pattern in monotocous species such as humans and cattle [1–3], but the intrafollicular mechanisms involved are not fully understood. Members of the transforming growth factor-β (TGFβ) superfamily of ligands, which includes the bone morphogenetic protein (BMP) subfamily, have been shown to have a species-specific functional role in follicular development and granulosa cell function in numerous species. Mice with inactivating mutations in the *Gdf9* gene have normal numbers of primordial follicles, but follicular growth is blocked at the primary stage, which implies that GDF9 plays a role in the transition of primary to secondary follicles [4]. Mice null for the *Bmp15* gene are subfertile [5], whereas mice that overexpress BMP15 have accelerated follicular growth and an increase in secondary follicles but normal litter sizes [6]. In contrast, sheep with inactivating mutations in *Bmp15*

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http://dx.doi.org/10.1016/j.domaniend.2012.10.002

have normal numbers of primordial follicles but abnormal primary follicle development (reviewed in McNatty et al., [7]). Similarly, immunization against either BMP15 or GDF9 in sheep impedes follicular development beyond the primary follicle stage [8]. Active immunization studies in cattle also support a functional role for GDF9 and BMP15 in follicular development [9].

Although the ability of GDF9 and BMP15 to regulate granulosa cell steroidogenesis has been established in multiple species, including cattle [10,11], additional TGFβ family members, beyond the oocyte-specific ones, have also been shown to regulate granulosa cell steroidogenesis in vitro. Treatment with BMP4, BMP6, and BMP7 increased basal and IGF1-induced estradiol production by bovine granulosa cells in vitro [12], and decreased forskolin-induced progesterone production in luteinized bovine granulosa cells in vitro [13]. Previous studies that used ovaries of abattoir origin indicated BMP2 is selectively expressed within the granulosa layer of bovine follicles [14]. However, the temporal regulation of expression of *Bmp2* and its type I and type II receptors (*Bmpr1a* and *Bmpr2*) at specific stages of a follicular wave and the ability of BMP2 to regulate bovine granulosa cell estradiol production have not been conclusively established.

Evidence indicates cocaine- and amphetamine-regulated transcript (*Cartpt*), is a novel intraovarian regulator of follicular development in cattle. The mature *Cartpt* peptide (CART) is a potent negative regulator of FSH-induced [15,16] and IGF1-induced [17] estradiol production in vitro and can inhibit follicular estradiol production in vivo [18]. Follicular fluid CART concentrations in healthy follicles decrease after dominant follicle selection, and *Cartpt* mRNA is lower in healthy vs atretic follicles collected before and early after initiation of follicle dominance, suggestive of a regulatory role in the selection process [18]. The inhibitory actions of CART on FSH signaling and estradiol production depend on the $G_{o/i}$-subclass of inhibitory G proteins and are linked to multiple components of the FSH signal transduction pathway, resulting in reduced *Cyp19A1* mRNA and estradiol production [15,16]. However, the ability of CART to affect bovine granulosa cell estradiol production mediated by other trophic factors implicated in regulation of follicular development has not been established. Hence, the objectives of the present study were to determine 1) the temporal regulation of mRNA for *Bmp2* and its receptors (*Bmpr1a*, *Bmpr2*) at specific stages of a follicular wave in cattle, 2) the effect of BMP2 on bovine granulosa cell steroidogenesis in vitro, and 3) the ability of CART to modulate potential effects of BMP2 on granulosa cell steroidogenesis.

2. Materials and methods

2.1. Animal procedures, ovary collection, and sample processing

Details on ultrasound monitoring of follicular growth, ovary collection, and RNA isolation and reverse transcription for bovine granulosa cells from follicles from ovaries collected at specific stages of a follicular wave were published previously [18]. Briefly, all animal procedures were approved by the Michigan State University Institutional Animal Care and Use Committee. Ovaries were removed during the following stages of the first wave of the estrous cycle: predeviation (1.5 d after emergence or first day a follicle >4 mm is detected by ovarian ultrasonography; n = 4), early dominance (first day in wave when 1 follicle in the cohort is 2 mm larger than others; n = 3), mid dominance (second day in wave that the dominant follicle does not increase in size; n = 5), and from an ovulatory wave (preovulatory; 1 d after prostaglandin F2α injection on day 7 after estrus; n = 6). Granulosa cells were then isolated from the F1 (largest follicle) from each animal, and RNA isolation and reverse transcription were performed as previously described [15].

2.2. Real-time PCR

Real-time PCR was performed as previously described [15,18]. Primers were designed with either PerlPrimer [19] or Primer express (Applied Biosystems, Carlsbad, CA, USA) and contained the following sequences: *Bmp2* forward, 5′-AAGGCCCTTGCTTGTCACTTT-3′, and reverse, 5′-TGCTTG CCGCTTTTCTCTTC-3′; *Bmpr1a* forward, 5′-TCAGCGAACTA TTGCCAAACAG-3′, and reverse, 5′-CCCATCCACACTTCTCCG TATC-3′; *Bmpr2* forward, 5′-AACACCACTCAGTCCACCTC-3′, and reverse 5′-GTCAGCATCCTATATCCAAAGCA-3′; and *Rps18* forward, 5′-GTGGTGTTGAGGAAAGCAGACA-3′, and reverse, 5′-TGATCACACGTTCCACCTCATC-3′. Relative expression levels for the target genes of interest (*Bmp2*, *Bmpr1a*, *Bmpr2*) were calculated with the ΔΔCT method [20], using *Rps18* as the housekeeping gene.

2.3. Granulosa cell culture

Granulosa cells were isolated from ovaries of abattoir origin at random stages of the estrous cycle, and 7-d serum-free granulosa cell culture was performed with previously described culture system in which cells display increased estradiol production with time in culture and respond to FSH with a dose-dependent increase in estradiol [15]. Briefly, granulosa cells were isolated from 3- to 5-mm follicles and pooled. The granulosa cells were washed 3 times in media (MEMα; Invitrogen, Carlsbad, CA, USA) containing insulin, IGF1, transferrin, sodium selenite, androstenedione (all from Sigma, St Louis, MO, USA), penicillin, streptomycin, and fungizone (Invitrogen). Granulosa cells (100,000 live cells per well) were plated in 96-well culture dishes (BD Biosciences, San Jose, CA, USA), and media were replaced every 2 d. The experiment was repeated 4 times, and treatments (in absence of FSH) consisted of media alone or media containing 10, 50, or 100 ng/mL recombinant human BMP2 (R&D Systems, Minneapolis, MN, USA) for 6 d. On the sixth day of culture, cells were treated for 24 h with the previous doses of BMP2 alone or in combination with 0.1 or 1 μM CART 55-102 (American Peptide Co, Sunnyvale CA, USA; 6–8 wells per treatment). After 24 h, media was removed and stored at −20°C until analysis for estradiol and progesterone concentrations, and the cells were washed, trypsinized, and counted as previously described [15].