Bone Morphogenetic Protein-15 Inhibits Follicle-stimulating Hormone (FSH) Action by Suppressing FSH Receptor Expression*

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We have recently reported that oocyte-derived bone morphogenetic protein-15 (BMP-15) can directly modulate follicle-stimulating hormone (FSH) action in rat granulosa cells. Here, we investigate underlying mechanisms of this BMP-15 effect. Treatment with BMP-15 alone exerted no significant effect on the basal expression of mRNAs encoding steroidogenic acute regulatory protein, P450 side chain cleavage enzyme, P450 aromatase, 3β-hydroxysteroid dehydrogenase, luteinizing hormone receptor, and inhibin/activin subunits. However, BMP-15 markedly inhibited the FSH-induced increases in these messages. In striking contrast, BMP-15 did not change the forskolin-induced levels of these transcripts. Thus, the inhibitory effect of BMP-15 on FSH action must be upstream of cAMP signaling. We next examined changes in FSH receptor mRNA expression. Interestingly, BMP-15 severely reduced the levels of FSH receptor mRNA in both basal and FSH-stimulated cells. To determine whether this effect was at the level of FSH function, we investigated the effect of BMP-15 on FSH bioactivity. Consistent with the mRNA data, BMP-15 inhibited the biological response of FSH, but not that of forskolin. Based on these results, we propose that BMP-15 is an important determinant of FSH action through its ability to inhibit FSH receptor expression. Because FSH plays an essential role in follicle growth and development, our findings could have new implications for understanding how oocyte growth factors contribute to folliculogenesis.

FSH is an essential for normal folliculogenesis and female fertility (1, 2). In the ovary, FSH interacts with its receptor on the granulosa cells (GCs) to initiate cytodifferentiation and proliferation that ultimately result in the development of pre-ovulatory follicles (3, 4). The mechanism of FSH action involves the activation of specific genes in the GCs through the cAMP-dependent protein kinase-A signaling pathway (5–7). Some of the physiologically important genes that are induced by FSH signaling include the P450 aromatase (P450arom) and luteinizing hormone receptor (LH-R), steroidogenic acute regulatory protein (star), P450 side chain cleavage enzyme (P450sc), 3β-hydroxy steroid dehydrogenase (3β-HSD), inhibin, and activin (5, 8). The physiological importance of FSH action is demonstrated by the fact that, when restricted, the developing follicles die by apoptosis, and there are no ovulations (9, 10). Therefore, it is important to define the mechanisms involved in FSH action.

There is a large body of evidence indicating that oocytes secrete factors that modulate FSH action (11–13). In vitro experiments have shown that oocyte-derived factors can act to inhibit FSH-induced expression of P450scc (14), progesterone (P4) production (14), urokinase plasminogen activator (15), and LH-R mRNA (16), while acting to stimulate mitosis (17), hyaluronic acid (18–20), and estradiol (E2) production (21). There is compelling evidence that growth and differentiation factor-9 (GDF-9) is one oocyte factor involved in regulating these proliferative and differentiation responses (22–28).

Recently, attention has been focused on another oocyte growth factor involved in regulating FSH action: the new member of the transforming growth factor-β superfamily designated as bone morphogenetic protein-15 (BMP-15) or growth and differentiation factor-9 (GDF-9) (29–31). The cloning of the cDNAs in the mouse (29), human (29), and rat (32) has revealed that the primary structure of BMP-15 is most closely related to that of GDF-9 (29, 32). To date the only cell type known to express BMP-15 is the mammalian oocyte (29–31). Regarding function, we have recently reported that BMP-15 stimulates rat GC proliferation and selectively inhibits FSH-induced P4, but not E2, production (33). This is the only evidence available to date concerning the function of the BMP-15 molecule.

The potential physiological relevance of BMP-15 in vivo is beginning to emerge. A recent genetic study of a naturally occurring mutant in sheep termed Inverdale (FecX) has provided evidence for an essential role of BMP-15 in folliculogenesis and fertility (34). In the female homozygous FecX mutants, follicular development arrests at the primary stage, resulting in infertility. This phenotype has been linked to a defect in the BMP-15 gene. By contrast, the heterozygotes exhibit increased ovulation rate and multiple pregnancies. Therefore, BMP-15 is associated with the mechanisms of infertility and super fertility in a dosage-sensitive manner (34). An important unanswered question is how BMP-15 regulates these physiological functions.

The present study provides the evidence that BMP-15 inhibits major FSH actions that are obligatory for follicle development and ovulation by virtue of its ability to suppress FSH.
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**EXPERIMENTAL PROCEDURES**

**Reagents and Supplies—**Ovine FSH (NIDDK-oFSH-S20) was provided by the National Hormone and Pituitary Program (Rockville, MD). Diethylstilbestrol, forskolin, and 4-androstene-3,17-dione (androstenedione, substrate of P450arom) were purchased from Sigma and female Harlan Sprague-Dawley rats (23 day old) from Charles-River Lab. (Wilmington, MA). Recombinant human BMP-15 tagged with a FLAG-epitope (BMP-15) was produced by 293 cells and purified by anti-FLAG monoclonal antibody as reported previously (33).

**Primary Cell Culture—**Female Harlan Sprague-Dawley rats (23 days old) were implanted with silastic capsules containing 10 mg of diethylstilbestrol to increase GC number (3). After 4 days of diethylstilbestrol exposure, GCs were collected from the ovaries and cultured in serum-free McCoy's 5a medium supplemented with 2 mM l-glutamine and antibiotics as described previously (35). The animal protocols were approved by the University of California at San Diego Institutional Animal Care and Use Committee.

**RNA Extraction and Analysis by Quantitative Competitive RT-PCR—**GCs (2 × 10^5 viable cells) were cultured in a six-well plate with 2 ml of McCoy's 5a medium containing one or a combination of the following: 10 ng/ml FSH, 100 ng/ml BMP-15, 10 μM forskolin. One-hundred nM androstenedione was added to the culture medium as indicated. After 48 h culture, total RNA was extracted by guanidium isothiocyanate-phenol-chlorform methods using TriZOL® (Life Technologies Inc.), quantified by measuring absorbance at 260 nm, and stored at −80 °C until assay. Oligonucleotides used for RT-PCR were custom-ordered from Life Technologies, Inc. PCR primer pairs were selected from different exons of the corresponding genes to discriminate PCR products that might arise from possible chromosome DNA contamination. Specifically, they are derived from the cDNA clones at the following nucleotide numbers: 1651–1670 and 1751–1770 for STAR (36); 148–167 and 637–656 for P450arom (38); 317–336 and 437–456 for 3β-HSD (39); 524–543 and 684–703 for 20α-HSD (30); 428–447 and 588–607 for inhibin βA-subunit (239–258 for inhibin A-subunit; 428–447 and 588–607 for inhibin βA-subunit; 32–51 and 239–258 for inhibin β-subunit (34); 401–421 and 575–595 for ribosomal protein-L19 (L19) (44). The steady state levels of mRNA encoding STAR, P450scc, P450arom, and FSH-R (referred to “target” mRNA) were analyzed by quantitative competitive RT-PCR. Prior to PCR the internal control DNAs (~340 base pairs) having target-specific primer pairs were generated by PCR as reported previously (45). The extracted RNA (500 ng) was subjected to a RT reaction using a First-Strand cDNA Synthesis System (Life Technologies, Inc.) with random hexamer (2 ng/μl), reverse transcriptase (200 units), and deoxynucleotide triphosphates (dNTP; 0.5 mM) at 42 °C for 50 min, 70 °C for 10 min. The resultant single strand cDNA was resuspended in 50 μl of water for competitive PCR. The linear portion of the relationship between target cDNAs and internal control DNAs was determined individually for all target mRNAs. For this, a fixed amount (20 μl of cDNA derived from GCs treated with FSH was mixed with a series of 10-fold dilutions of the internal control DNA, and the target and the internal control DNAs were amplified by PCR using a specific primer set for the individual target (Fig. 1). Competitive PCR was performed using MgCl2 (1.5 mM), dNTP (0.2 mM), and 2.5 units of Platinum Taq DNA polymerase (Life Technologies, Inc.) under the following conditions: 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. Aliquots of PCR products were electrophoresed on 2% agarose gels, visualized after ethidium bromide staining, photographed, and scanned. The relative integrated density of each band was digitzed by multiplying the absorbance of the surface area (NIH image 1.62). Finally, the ratios of the densitometric readings of the amplified target cDNA and internal control DNA were plotted on the ordinate against the serial 10-fold dilutions of internal control DNA on the abscissa (Fig. 1).

**RESULTS**

In previous experiments (33) we found that BMP-15 inhibits FSH-induced P4, but not E2, production by cultured rat GCs. To test the hypothesis that BMP-15 may be specifically involved in modulating the expression of steroidogenic genes in response to FSH stimulation, we analyzed mRNA levels for STAR, P450scc, and P450arom by competitive reverse PCR.

As shown in Fig. 2, treating GCs for 48 h with a saturating dose of BMP-15 had no effect on the steady state mRNA levels for STAR, P450scc, P450arom, and FSH-R. To examine the mRNA levels of BMP-15, we treated GCs with BMP-15 and analyzed the mRNA levels for BMP-15 (10 ng/ml) and BMP-15 (100 ng/ml) by quantitative competitive RT-PCR. As shown in Fig. 3, forskolin (10 μM)
stimulated the levels of StAR, P450scc, and P450arom mRNAs similar to that seen with FSH. However, in striking contrast to the FSH results, BMP-15 failed to change the forskolin-induced mRNA levels of these steroidogenic factors. L19 mRNA levels had no change by the forskolin treatment regardless of the presence of BMP-15 among four groups. These results suggest that the inhibitory effect of BMP-15 on FSH-induced StAR and P450scc mRNA expression is mediated by a pre-cAMP signaling event.

The failure of BMP-15 to inhibit FSH-induced P450arom mRNA expression is paradoxical. Given that androgens can up-regulate FSH-induced P450arom gene activity (47, 48), we wondered whether the androstenedione in the culture medium could explain this paradox. As seen in Fig. 4, FSH stimulated P450arom mRNA expression in the absence of androstenedione and importantly, this increase was significantly inhibited by BMP-15. As expected (see Fig. 3), the forskolin-induced P450arom expression was not changed by BMP-15 in the absence of androstenedione. The steady state mRNA levels of StAR, P450scc, and L19 were unaffected by androstenedione (data not shown). Thus, it is clear that in the absence of androstenedione, P450arom mRNA induced by FSH was also inhibited by BMP-15 at a site upstream of the cAMP signaling event.

To investigate whether the BMP effect involves changes in FSH-R expression, we measured the mRNA levels for FSH-R by quantitative competitive PCR. First, we established a linear relationship between the expression of target FSH-R and internal control (Fig. 5A) and then selected 10^2 pM concentration of internal control to perform the competitive reactions. Control untreated cells expressed FSH-R mRNA spontaneously. Interestingly, the level of FSH-R mRNA in control cells was markedly reduced (80%) following treatment with BMP-15 (Fig. 5B). FSH treatment increased FSH-R mRNA levels 2-fold, and this increase was completely abolished by cotreatment with BMP-15, actually decreasing the FSH-R mRNA to that seen with BMP-15 alone. Thus, both basal and FSH-induced FSH-R mRNA expression is negatively regulated by BMP-15.

To determine whether BMP-15 modulates other FSH-dependent functions in GCs, we examined the possible role of BMP-15 in the regulation of LH-R, the inhibin subunits (α, βA, and βB), 3β-HSD, and 20α-HSD. As seen in Fig. 6, FSH induced marked increases in the levels of mRNA for each of these end points, except for 20α-HSD. Although BMP-15 alone had no effect on the basal levels of these mRNAs, it totally abolished the stimulatory effects seen with FSH (Fig. 6). BMP-15, either alone or together with FSH, did not change the basal levels of 20α-HSD mRNA. As illustrated in Fig. 7, forskolin induced the expression of high levels of the mRNAs encoding LH-R, inhibit α, βA and βB subunits, and 3β-HSD. Consistent with our earlier results with StAR, P450scc and P450arom, cotreatment with BMP-15 failed to alter the forskolin-induced expression of these mRNAs (Fig. 7).

To determine whether this BMP-15 inhibition was at the level of FSH function, we tested the effect of BMP-15 on FSH bioactivity, specifically, P4 and E2 production in the GCs. In

FIG. 2. Effects of FSH and BMP-15 on the expression of the mRNAs for StAR, P450scc, and P450arom. GCs were cultured either alone or together with FSH (10 ng/ml) and/or BMP-15 (100 ng/ml) in the presence of androstenedione (100 nM) for 48 h, after which total RNA was extracted and then subjected to quantitative competitive RT-PCR analysis as described under “Experimental Procedures.” The PCR products are shown in insets, and the ratios of PCR products (target/ internal control) are graphed. Bars with different letters indicate that group means are significantly different at p < 0.01.

FIG. 3. Effect of forskolin (10 μM) and BMP-15 (100 ng/ml) on the expression of the mRNAs for StAR, P450scc, and P450arom. The experimental design is as described in the legend to Fig. 2.
agreement with our recent study (33), FSH-induced P4, but not E2, production was suppressed by BMP-15 (Fig. 8). In contrast, the forskolin-induced steroidogenic responses were unaffected by BMP-15 (Fig. 8). These results fit our hypothesis that BMP-15 inhibits FSH action by suppressing functional FSH-R in GCs.

**DISCUSSION**

In a recent study we showed that BMP-15 stimulates proliferation of rat GCs and negatively regulates FSH-induced P4, but not E2, production in vitro (33). The objective of the present study was to begin to explore the molecular basis by which BMP-15 modulates FSH action. The major new findings of the present study are as follows. First BMP-15 reduced the steady state levels of mRNA induced by FSH, including StAR, P450scc, 3β-HSD, LH-R, inhibin/activin subunits, and FSH-R. These observations suggest that BMP-15 can act broadly to inhibit the expression of a large battery of genes induced by FSH. Accordingly, BMP-15 can be considered a negative regulator of the major actions of FSH in the rat ovary. Second, in striking contrast to the FSH action, BMP-15 did not affect the stimulation of this battery of mRNAs in response to forskolin. Therefore, one can conclude that BMP-15 exerts its inhibitory effect on FSH action upstream of cAMP signaling. Third, BMP-15 markedly decreased both the basal and FSH-induced increases in FSH-R mRNA. And fourth, FSH-induced, but not forskolin-induced, steroidogenesis was suppressed by BMP-15, a finding that is consistent with the decrease in FSH-R message. Based on these findings, we propose that the negative regulation of FSH-R expression is the primary cause of BMP-15 inhibition of FSH action. It is important to note, however, that because we did not measure FSH protein levels directly, we...
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It was found that BMP-15 does not inhibit the amplifying effect of androstenedione on FSH-stimulated P450arom mRNA expression. But, it did suppress the ability of FSH to induce P450arom in the absence of androstenedione. This is consistent with the failure of BMP-15 to inhibit FSH-induced E2 production in the presence of added androstenedione. The potential significance of this finding is unknown. In this regard, however, a fundamental principle in ovarian physiology is that FSH stimulates the expression of P450arom and E2 production in the dominant follicle that contains a high concentration of androstenedione in the follicular fluid. Therefore, the finding that BMP-15 does not inhibit FSH-induced P450arom mRNA and E2 production in the presence of androstenedione may be physiologically relevant.

Genetic and physiological studies in Inverdale (FecX1) sheep have identified a point mutation in the BMP-15 gene that has profound effects on follicle development and ovulation quota (34). Homozygous FecX1 mutant females are infertile because follicle growth is arrested at the primary preantral stage. Consequently there are no ovulations in these animals. This provides compelling evidence for a requirement of bioactive BMP-15 in follicle cell proliferation and differentiation in sheep (51). Surprisingly, the heterozygous FecX1 mutants exhibit increased ovulation rate. In this connection, several interesting abnormal features of the ovaries of the heterozygotes have been identified: (i) there are more healthy estrogenic follicles, ii) the number of GCs in these developing follicles is significantly smaller, iii) these GCs have a higher mean LH responsiveness at smaller follicle stages, and iv) the corpora lutea are smaller (52). The fact that the plasma FSH and LH levels in the heterozygotes are normal would imply that the mechanisms responsible for these unusual features in the heterozygote reside in the ovary. Our observation that BMP-15 inhibits FSH-R expression could explain the cause of the abnormal phenotype of the Inverdale ewe. In the heterozygotes, we propose that reduced levels of BMP-15 result in higher levels of FSH-R in the GCs, which in turn lead to developing healthy estrogenic follicles with more LH-R. The end result of this sequence of events would be increased ovulation rate. At the opposite extreme, follicle development in the homozygotes is arrested at the primary follicle (FSH-independent) stage. In these animals we propose that the absence of BMP-15 results in the cessation of GC proliferation, which in turn leads to arrested follicle development.

In summary, the conclusion emerging from these findings is that BMP-15 can inhibit some of the most important events in the process of FSH-dependent GC cytodifferentiation, including the expression of the FSH-R itself. Consequently the oocyte can play an important role in determining GC proliferation and FSH sensitivity in developing follicles.

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