Dynamic regulation of pituitary mRNAs for bone morphogenetic protein (BMP) 4, BMP receptors, and activin/inhibin subunits in the ewe during the estrous cycle and in cultured pituitary cells

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Abstract

Recently, bone morphogenetic protein (BMP) 4 has been shown to inhibit FSH secretion in ewe. The detection of BMP4 mRNA and BMP receptors in the pituitary suggests that BMP4 can exert paracrine actions on FSH production. This work aimed at determining whether BMP4 and/or BMP receptor mRNA as well as activin/inhibin subunit mRNA fluctuates during the estrous cycle when FSHβ mRNA and FSH release changed. The estrous cycles of ewes were synchronized with progestagen sponges. Ewes were killed in late follicular stage (n = 5), before the secondary FSH surge (n = 4), and in luteal phase (n = 4). Using quantitative reverse transcription-PCR, we showed that the levels of mRNA for BMP4, BMP receptor, the inhibitor of differentiation 2 (Id2), a target gene of BMP4, and noggin did not change significantly across the estrous cycle. In contrast, the level of activin βB mRNA and the percentage of immunoreactive cells for activin βB-subunit were higher before the secondary surge of FSH compared to other groups. In ewe pituitary cell cultures, activin, GnRH, or estradiol-17β (E2) did not significantly affect the levels of BMP4, BMP receptor, and Id2 mRNA. E2, but not GnRH, increased the level of activin βB mRNA. Moreover, the in vitro FSH release was not modified by noggin, a BMP antagonist. In contrast, SB431542, an inhibitor of activin pathway, inhibited FSH release. Collectively, our data showed that pituitary BMP4 would not play a crucial role in the regulation of FSH production during the estrous cycle, whereas local activin B would be a major stimulus of FSH synthesis necessary for the secondary FSH surge.

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Introduction

In mammals, the gonadotropins FSH and LH are essential components for reproduction (Morel et al. 1981). Synthesis and release of LH and FSH by gonadotrope cells are regulated by a complex interplay of hypothalamic, gonadal, and pituitary factors. The hypothalamic GnRH is the major regulator of LH and FSH secretion. While the LH secretion is mainly dependent of GnRH and gonadal steroids, additional factors are involved in regulation of FSH production. Particularly, activins and inhibins, members of the transforming growth factor-β (TGF-β) superfamily, are able to stimulate (Ling et al. 1986) and inhibit (Carroll et al. 1989) FSH synthesis and release respectively. Activin is a dimer of two highly related β-subunits (βA and/or βB), resulting in three possible molecular species: activin A (βA βA), activin B (βB βB), and activin AB (βA βB). Inhibin is a heterodimer composed of one α-subunit and one β-subunit (βA or βB). The activin and inhibin subunits are produced in both ovary and pituitary gland in different species, including rat (Kogawa et al. 1991, Wilson & Handa 1998), human (Uccella et al. 2000), and sheep (Baratta et al. 2001). Activin is thought to play an autocrine/paracrine action on FSH secretion (Padmanabhan & McNeilly 2001). Indeed, monoclonal antibodies to activin B inhibit FSH release from plated pituitary cells as well as from hypophysectomized, pituitary-grafted rats (Corrigan et al. 1991, DePaolo et al. 1992). Inhibin is produced by gonads and exerts an endocrine action on FSH secretion (de Kretser & Robertson 1989), mainly through antagonism of activin action (Lewis et al. 2000, Bilezikjian et al. 2004).

Recently, other members of the TGF-β superfamily, the bone morphogenetic proteins (BMPs), were shown to regulate FSH production in vitro. In rat pituitary cells and murine LβT2 gonadotrope cell lines, BMP6 and BMP7 at high concentrations or BMP2 and BMP15 at lower concentrations stimulate basal FSH secretion and FSHβ promoter activity (Huang et al. 2001, Otsuka & Shimasaki 2002, Lee et al. 2007). Moreover, BMP4 increased the release of FSH in response to activin and activin+GnRH.
BMPR1A (ALK3) and BMPR2 are immunodetected on ActR2B fluctuates across the estrous cycle (Fafioffe et al. 2005). In order to gain an insight into potential intrapituitary role of BMP4 in the regulation of FSH synthesis and release, the present study was designed to determine whether changes in the patterns of FSHβ gene expression and FSH secretion observed in vitro and in vivo during the estrous cycle were associated with variations in the expression of the BMP4 system, i.e. ligand, BMP receptors, noggin antagonist, and Id2, a target gene for BMP4. In the absence of available antibodies and protein immunoassays for ovine BMPs, we focused on mRNA levels. To compare the BMP system to those of activin, the pattern of activin subunit expression in the pituitary during the estrous cycle and in vitro was investigated.

Materials and Methods

Reagents

Cell culture reagents used were DMEM and F12 (nutrient mixture F-12 Ham) from Sigma. FCS and BSA were purchased from Sigma. Collagenase A and DNase I were obtained from Roche Diagnostics Ltd. Human recombinant activin-A and BMP4 were obtained from R&D Systems (Lille, France). Estradiol-17β (E2) and SB431542 were obtained from Sigma. GnRH (Relefact LH-RH) was obtained from Aventis (Frankfurt, Germany). Human noggin was kindly provided by Regeneron (Tarrytown, NY, USA).

Pituitary collection during the estrous cycle

The estrous cycles of 15 Ile de France ewes were synchronized by insertion of vaginal sponges impregnated with synthetic prostegestagen during the breeding season. Sponges remained in place for 12 days. Ewes were killed at different stages of the estrous cycle: 36 h (group A), 48 h (group B), 66 h (group C), or 240 h (group D) after sponge removal. Prior to killing, blood samples were collected from the jugular vein at 2 h intervals between 32 and 36 h for group A, 32 and 48 h for group B, and 32 and 66 h for group C. For group D, a single blood sample was collected just prior to killing. Plasma was recovered from these samples and assayed for LH, FSH, and E2. Anterior pituitaries were collected immediately after slaughtering and hemisected midsagittally. One half of pituitary was stored at −80 °C until RNA extraction, and the other half was fixed for 72 h in Bouin-Holland fixative containing HgCl2 for immunohistochemistry as previously described (Taragnat et al. 1998). All procedures were approved by the Agricultural and Scientific Research agencies, and conducted in accordance with the guidelines for Care and Use of Agricultural Animals in Agricultural Research and Teaching.

Pituitary collection for cell culture

Pituitaries from Ile de France ewes were collected during the non-breeding season and dissociated for cell cultures as previously described (Faure et al. 2005). Pituitaries were finely sliced and placed in F12 supplemented with collagenase A (0·4 mg/ml) and DNase I (0·025 mg/ml), and then incubated for 1 h 30 min in a shaking water bath at 37 °C followed by manual dispersion through different sizes of syringe. Cells were then centrifuged at 100 g for 5 min, and the pellet was resuspended in culture medium (DMEM containing 5% FCS). Cells were plated in 6-well plates at 3 × 10^6 cells/well in 2 ml of culture medium (DMEM with 5% FCS), and allowed to attach for 2 days in a humidified atmosphere with 5% CO2 at 37 °C. Media were replaced with serum-free DMEM containing 0·1% BSA. One hour later, media were changed and replaced by DMEM−0·1% BSA alone (control) or supplemented with test substances at different concentrations (see Results). The choice of concentrations was based on our previous studies determining 10^-8, 10^-9, and 10^-10 M as optimal doses for GnRH, activin, and E2 respectively to regulate FSH secretion in vitro. Media were collected for measurement of secreted FSH by ELISA. Total RNA was extracted from cells for measurement of studied mRNA levels by quantitative reverse transcription (RT)-PCR.

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**Gonadotropin measurements by ELISA**

The concentrations of ovine gonadotropins in blood plasma or in culture cell media were determined using double-antibody ELISA for all experiments, as previously described by Faure et al. (2005) with a sensitivity of 0·1 ng/ml for ovine LH (oLH) and 0·4 ng/ml for ovine FSH (oFSH). For LH, the intra- and inter-assay coefficients of variation (CV) of the control averaged 4 and 10% respectively. The cross reaction with oFSH was 0·01%. For FSH, the intra- and inter-assay CV of the control were 2·6 and 2·1% respectively. The cross reaction with oLH was 0·07%.

**E2 measurements by RIA**

For steroid assays, 600 μl plasma was extracted with ethyl acetate–cyclohexane as described (Monniaux et al. 2008), and the dried extracts containing steroids were measured by RIA. E2 was assayed with the E2 RIA kit following the manufacturer’s specifications (Diasorin SA, Antony, France). The limit of detection of the assay was 0·08 pg per tube. The intra- and inter-assay CV of the control were 3·8 and 8·7% respectively.

**Quantitative RT-PCR from tissue and from pituitary cell cultures**

Levels of mRNA for BMP4, BMP receptors, Id2, inhibin α-subunit, activin βA- and activin βB-subunits were measured using SYBR Green RT-PCR. Total RNA from 6-well plates (plated at 3·10^6 cells/well) and from tissue was extracted using TRI Reagent (Sigma) and kit NucleoSpin RNA L (Macherey-Nagel, Hoerdt, France) respectively according to the manufacturer’s instructions. Remaining DNA was removed by RNase-free DNase treatment. cDNA was synthesized from 1 μg RNA in a volume of 20 μl containing 150 ng oligo(dT) (Promega), 1 mM dNTPs, 20 U of RNasin, 1× RT PCR buffer, and 12 U M-MLV reverse transcriptase (Promega). For the negative control, the reverse transcriptase was omitted. The RNA denaturation was performed at 70 °C for 10 min, and the RT was performed at 37 °C for 1 h.

For PCR, each reaction consists of a final reaction volume of 20 μl containing iQ SYBR Green Supermix (Bio-Rad) (1×), 3 μM of each gene-specific primer, and cDNA. Primers were designed using Beacon Designer Software (Premier Biosoft International, Palo Alto, CA, USA), and sequences are described in Table 1. The equivalent of 5–50 ng of starting RNA was used in each reaction. Each sample was assayed in duplicate. Quantitative real-time PCR was run on an iCycler from Bio-Rad. The amplification program consisted of 95 °C for 30 s followed by up to 40 cycles of 95 °C for 30 s, 58–61 °C for 30 s, and 72 °C for 20 s. Annealing temperatures are given in Table 1. Prior to analysis, amplification efficiency was determined for each gene by generating a standard curve using serial dilutions of the cDNA, obtained after reverse transcriptase of the RNA, in abscissa and the corresponding cycle

<table>
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<th>Primer</th>
<th>GenBank</th>
<th>Sequence</th>
<th>Annealing temperature (°C)</th>
<th>Product size (bp)</th>
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<td>L19</td>
<td>AY158223</td>
<td>5′-AATCGCCAATGCCAACTC</td>
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<td></td>
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<td>3′-CCCTTTCGCTTACCTATACC</td>
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<td>FSHβ</td>
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<td>3′-GGTTTGTCTGCGGGAGATGC</td>
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<td>Noggin</td>
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<td>3′-ACGGAGGTTGAGGCTGTC</td>
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<td>Activin βB (tissue)</td>
<td>FJ167874</td>
<td>5′-GACAGCAGGCACCGCAGCT</td>
<td>59</td>
<td>148</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3′-CCGCAATGGTCCCGTATAGC</td>
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<td>Activin βB (cell)</td>
<td>FJ167874</td>
<td>5′-GCGGAAAGGTGCGGGAAGG</td>
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<td>Inhibin α</td>
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<td></td>
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threshold (C) in ordinate. The slope of the log-linear phase reflects the amplification efficiency derived from the formula \(E = (10^{-\text{slope}} - 1) \times 100\). Amplification efficiency was included between 95 and 105%, and was equivalent for test gene and the reference gene. Amplification was followed by melting curve analysis for each primer pair to verify the presence of one gene-specific peak and the absence of primer dimers. PCR products obtained with each set of primers were run on an agarose gel, and visualized using ethidium bromide and u.v. illumination to confirm amplification of a single product of the correct size. This was followed by sequencing of each amplicon to confirm the identity of amplified product, and the results were matched with the National Center for Bioinformatics Computing (NCBI-BLAST) database. Quantification of specific mRNA levels was performed by the \(\Delta \Delta C\) method with the internal reference gene \(L19\) (Bulletin #2; PE Biosystems, Foster City, CA, USA). The intra- and inter-assay CV for tissue analyses were ranged between 1.3 and 6.6% and between 1.9 and 10.8% respectively. The intra- and inter-assay CV for cell analyses were 1.5–6.9 and 3.4–9.7% respectively.

**Immunocytochemistry**

Affinity-purified polyclonal antisera raised in rabbits against synthetic fragments of porcine inhibin \(\alpha\) (1–26)-Gly–Tyr, \(\beta A\) (81–113)-NH\(_2\), or \(\beta B\) (80–112)-NH\(_2\) were kindly provided by W Vale and characterized previously (Vaughan et al. 1989). Pituitary sections (7 μm) were incubated for 20 min in 0.3% hydrogen peroxide, rinsed with 0.1 M PBS (NaHPO\(_4\), Na\(_2\)PO\(_4\), and 9 g NaCl/l), and exposed to avidin–biotin peroxidase complex (DAKO, Trappes, France) for 30 min. The sections were then treated for 8 min with 0.04% 3,3′-diaminobenzidine tetrahydrochloride dihydrate (Sigma) and 0.03% H\(_2\)O\(_2\) in Tris–HCl (0.05 M, pH 7.8). Sections were coveredslipped with Depex. All incubations were carried out in a humidified chamber at room temperature. Specificity of the staining was confirmed since immunostaining was abolished when non-immune rabbit serum was substituted for the primary antibody. Cells immunostained for \(\alpha\)- or \(\beta B\)-subunit and cells that were immunonegative were counted at X40 objective after counterstaining with hematoxylin. Data were calculated by counting ~1000 total cells from three randomly selected sections per animal in an immunohistochemistry assay. Two immunohistochemistry assays were performed per animal. The percentage of immunoreactive cells within the total cells was calculated as an average of the two immunohistochemistry assays for each animal.

<table>
<thead>
<tr>
<th>Late follicular</th>
<th>Before 2nd FSH surge</th>
<th>Luteal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma E(_2) (pg/ml)</td>
<td>4.7±0.99</td>
<td>1.02±0.26</td>
</tr>
<tr>
<td>Plasma LH (ng/ml)</td>
<td>0.45±0.10</td>
<td>1.02±1.30</td>
</tr>
<tr>
<td>Plasma FSH (ng/ml)</td>
<td>0.28±0.12</td>
<td>0.64±0.20</td>
</tr>
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</table>

**Statistical analysis**

The levels of hormones and mRNAs during the estrous cycle and in cultured pituitary cells were compared using one-way ANOVA followed by the Newman–Keuls test. For estrous cycle experiment, two repetitions of RT-PCR using different

![Image](318x156 to 524x434)

**Figure 1** Changes in mRNA levels for FSHβ (A), BMP4 (B), BMPR1A (C), BMPR1B (D), BMPR2 (E), Id2 (F), and noggin (G) across the estrous cycle. Pituitaries were collected from ewes killed in late follicular phase (n=5), before the secondary FSH surge (n=4), and in luteal phase (n=4). Total RNA was extracted from the pituitaries of each animal, and 1 μg total RNA was analyzed by real-time RT-PCR and standardized by the level of L19 mRNA in each sample. Values are means ± S.E.M. a versus b, P<0.05.
RNA samples from different pieces of pituitary from the same animal of each group were performed separately. The mean of these two repetitions represented the value of each animal. For culture experiments, at least three experiments were performed in duplicate when RNA was analyzed or in triplicate when FSH concentrations were measured. All results are expressed as means ± S.E.M. Values of 0.05 were considered statistically significant. The statistical analyses were performed using GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA, USA).

Results

Plasma hormone concentrations during the estrous cycle

Plasma hormone concentrations and morphological analysis of ovaries allowed reclassification of animals into four groups. Thus, five ewes were killed before the preovulatory LH surge (group 1: late follicular, n = 5), two ewes during the preovulatory LH surge (group 2: LH surge, n = 2), four after ovulation (group 3: before the secondary FSH surge, n = 4), and four during the luteal phase (group 4: luteal, n = 4). Since there were only two animals in the LH surge group, we excluded this group from the following analyses. Plasma concentrations of E2 in blood samples recovered at the time of killing gave further evidence for the correct timing (Table 2).

mRNA expression of FSHβ during the estrous cycle

The mRNA levels for FSHβ-subunit in the pituitaries were determined by real-time PCR (Fig. 1A). Mean FSHβ-subunit mRNA levels varied during the estrous cycle. They increased before the secondary FSH surge compared to the late follicular phase (P<0.05).

mRNA expression of BMP4, BMP receptors, Id2, and noggin during the estrous cycle

As shown in Fig. 1B–E, BMP4 and BMP receptor mRNA levels did not significantly change in the different stages of the estrous cycle. Similarly, the levels of mRNA for the inhibitor of differentiation 2 (Id2) reflecting the BMP4 bioactivity in vitro, since they are stimulated by BMP4 (see later), were similar in all studied stages of the estrous cycle (Fig. 1F). Further, the mRNA level for the BMP4 antagonist, noggin, did not significantly change over the estrous cycle (Fig. 1G).

mRNA expression of activin βA-, activin βB-, and inhibin α-subunits during the estrous cycle

The mRNA levels for activin βA, activin βB, and inhibin α-subunits in the pituitaries were also evaluated by real-time PCR. The level of activin βB mRNA increased before the secondary FSH surge compared with the other stages of

Figure 2 Changes in mRNA levels for activin βA- (A), activin βB- (B), and inhibin α- (C) subunits throughout the ewe estrous cycle. Pituitaries were collected from ewes killed in late follicular phase (n = 5), before the secondary FSH surge (n = 4), and in luteal phase (n = 4). Total RNA was extracted from the pituitaries of each animal, and 1 μg total RNA was analyzed by real-time RT-PCR and standardized by the level of L19 mRNA in each sample. Values are the mean ± S.E.M. a versus b, P<0.05.

Figure 3 Immunostaining for inhibin α-subunit (A) and activin βB-subunit (B) on section from ewe pituitary collected before the secondary surge of FSH. Scale bars represent 18 μm. Percentages of immunoreactive cells for inhibin α-subunit (C) and activin βB-subunit (D) among the total pituitary cells during the estrous cycle. Pituitaries were collected from ewes killed in late follicular phase (n = 5), before the secondary FSH surge (n = 4), and in luteal phase (n = 4). Values are the mean ± S.E.M. a versus b, P<0.05. Full colour version of this figure available via http://dx.doi.org/10.1677/JOE-10-0035.
the cycle (Fig. 2B). In contrast, the amounts of activin βA or inhibin α mRNA were similar in all studied stages of the estrous cycle (Fig. 2A and C).

**Percentage of pituitary cells expressing activin βB- and inhibin α-subunits during the estrous cycle**

The presence of α- and βB-, but not βA-, subunits was confirmed by immunocytochemistry (Fig. 3A and B). The percentage of α-subunit immunoreactive cells did not vary throughout the estrous cycle (1.6 ± 0.2% of total pituitary cells; Fig. 3C). In contrast, the percentage of βB-subunit increased before the secondary FSH surge compared to the follicular phase (2.6 ± 0.2 vs 0.8 ± 0.1% of total pituitary cells, \( P < 0.005 \); Fig. 3D).

**Effect of GnRH, activin, and E2 on mRNA expression of BMP4, BMP receptors, and Id2 in cultured ovine pituitary cells**

To determine whether major factors of FSH regulation, such as GnRH, activin, or E2, were capable of modifying mRNA expression of BMP4 and/or BMP receptors, ovine pituitary cells were incubated with 10^{-8} M GnRH for 6 h, or 10^{-7} M activin or 10^{-8} M E2 for 48 h. Figure 4A shows that 6 h treatment with GnRH decreased the levels of FSHβ mRNA by 47% compared to basal conditions. Similar treatments with GnRH did not affect the mRNA levels for BMP4, BMPR1A (ALK3), BMPR1B (ALK6), BMPR2, and Id2 (Fig. 4B–F). When cells were treated for 48 h with activin or E2, the levels of FSHβ mRNA were increased by 29% \( (P < 0.05) \) or decreased by 63% \( (P < 0.05) \) respectively compared to basal conditions (Fig. 5A). There was no significant change in the mRNA levels for BMP4, BMPR1A (ALK3), BMPR1B (ALK6), BMPR2, and Id2 (Fig. 5B–F). However, in the presence of E2, the amount of mRNA for BMPR1B (ALK6) tended to increase compared to basal conditions \( (P < 0.1; \) Fig. 5B and D).

**Effect of GnRH and E2 on mRNA expression of activin βB in cultured ovine pituitary cells**

Treatment of pituitary cells with GnRH for 6 h did not significantly modify the amount of mRNA for activin βB-subunit (Fig. 4G). In contrast, treatment with E2 for 48 h increased the mRNA level \( (P < 0.05; \) Fig. 5G).

**Noggin does not affect FSH release**

If BMP4 acts on FSH synthesis through a paracrine or autocrine mechanism, we can expect that blocking its action with an antagonist leads to an increase in FSH secretion. We first checked that noggin, a BMP binding protein, which antagonizes BMP signaling, was able to prevent the action of exogenous BMP4 on FSH release. As shown in Fig. 6A, noggin (100 ng/ml for 24 h) blocked the inhibitory action of BMP4 (40 ng/ml) on FSH release in ovine pituitary cells. Similarly, it prevented the increase of Id2 mRNA level induced by BMP4 (Fig. 6B). However, noggin (from 10 to 100 ng/ml) did not significantly modify FSH concentrations in the culture media (Fig. 6C). Moreover, noggin alone did not affect the level of Id2 mRNA (Fig. 6B).
SB431542 affects FSH release

The activin receptor-like kinase (ALK) 4/5/7 inhibitor, SB431542, is able to prevent the action of exogenous activin on FSH release (Fig. 7A). Moreover, treatment with SB431542 from 0 to 10 μM for 24 h inhibited FSH secretion from pituitary cells in a dose-dependent manner (Fig. 7B). Preliminary analyses showed that the signaling capacity of BMP receptors assayed by the ability of BMP4 to induce Smad1 phosphorylation was not affected by SB431542 (data not shown).

Discussion

Several BMPs, in particular BMP4, are able to inhibit FSHβ mRNA expression and FSH release from ovine pituitary cells, whereas activin stimulates this secretion (Faure et al. 2005). The presence of BMP4 mRNA, as well as BMPR type IA (ALK3) and type II at the gonadotrope cell level in the adult pituitary, suggests that BMP4 can act within the pituitary and participate to control FSH synthesis and secretion in the ewe. A lot of studies raised the question of the mechanism of action of BMPs at the gonadotrope cell level, but no data are available concerning the possible changes of pituitary BMP system during the estrous cycle. To determine whether the variations in FSHβ mRNA and plasma FSH concentrations are associated with changes in the expression of BMP4 and BMP receptor mRNA as well as activin βB mRNA, three stages of the estrous cycle exhibiting variations of plasma FSH as previously shown (Leung et al. 1988, Fafioffe et al. 2004) were studied: late follicular phase, before the secondary FSH surge, and the luteal phase.

Our results showed that the levels of BMP4 mRNA in the pituitary did not significantly change between the studied stages of the estrous cycle when the level of FSHβ mRNA varied. In addition, the levels of BMPR1A (ALK3) and BMPR2 mRNAs, as well as those of BMPR1B (ALK6), were similar over the estrous cycle. Other receptors beside BMPR1A (ALK3), BMPR1B (ALK6), and BMPR2 can be involved in signal transduction. For example, ALK2

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BMP receptors, and activin in ewe pituitary

Figure 7 Effects of SB431542 (10 μM) and activin (40 ng/ml) on FSH release in ewe pituitary cells (A). Effect of increasing doses of SB431542 (0–10 μM) on FSH release in ewe pituitary cells (B). Cells were cultured in serum-free medium for 24 h in the presence of indicated concentrations of SB431542 and/or activin. Media were collected, and the concentrations of FSH were determined by ELISA. Values are the mean ± S.E.M. from three culture experiments with triplicate determinations. a versus b versus c, P<0.05.

(also known as Acvr1) is able to induce BMP signaling (ten Dijke et al. 1994, Macías-Silva et al. 1998, Lee et al. 2007) and was previously shown to increase at the secondary FSH surge in ewe (Fafioffe et al. 2004). Nonetheless, BMP4 and BMP2 are known to bind to BMPR1A (ALK3) and BMPR1B (ALK6), whereas other BMPs bind more strongly to ALK2 (ten Dijke et al. 1994, Miyazono et al. 2010). In the murine gonadotrope cell line LBT2, the endogenous signal-propagating type I receptor at least for BMP2, structurally close to BMP4, would be BMPR1A (Ho & Bernard 2009). BMPs also show promiscuity in their binding to type II receptor. For example, in the absence of BMPR2, BMP2 and BMP4 can use ActR2A (Yu et al. 2005). However, BMPR2 is present on the gonadotrope cells. Moreover, the level of ActR2A mRNA is similar throughout the estrous cycle (Fafioffe et al. 2004). All together, these data led us to study preferentially BMPR2, BMPR1A, and BMPR1B. The lack of variations in the expression of BMP4 and BMP receptor mRNA does not necessarily mean that pituitary BMP4 activity does not change. For example, in the rat, neither activin βB nor activin receptor mRNA expression changes at the time of the secondary FSH surge. Instead, the expression of follistatin, an activin binding protein, is modulated freeing up activins to stimulate FSH (Halvorsen et al. 1994). Hence, we followed the mRNA encoding noggin, a BMP4 antagonist. No change for the level of this mRNA was observed between the studied stages. We can argue that antagonists other than noggin can be involved. However, the absence of variation in the mRNA encoding Id2 (Peng et al. 2004), one BMP target gene reflecting the bioactivity of BMP4 in ewe pituitary cells, does not fit with this idea. All together, the data suggest that pituitary BMP4 activity did not significantly vary over the estrous cycle.

Because the levels of pituitary mRNAs during the estrous cycle result from a complex interplay between local and peripheral molecules, we examined the specific action of major factors of FSH regulation on the expression of BMP4, BMP receptors, and Id2 in cultured pituitary cells. Chronic treatment of pituitary cells with GnRH for 6 h, mimicking a situation similar to the preovulatory surge, induced a decrease in the level of FSHβ mRNA. This is consistent with the reduction observed in vivo in pituitaries collected during the LH surge (Fafioffe et al. 2004). Moreover, this agrees with reports showing that chronic GnRH inhibits the activity of the oFSHβ promoter and the rat FSHβ expression in vivo and in vitro (Attardi et al. 1989, Lerrant et al. 1995, Shafiee–Kermani et al. 2007). This inhibition can result from a fall of FSH mRNA stability and/or a decrease of the transcription. In contrast, chronic GnRH did not modify the amount of mRNA for BMP4, BMP receptors, and Id2.

Concerning the effect of the treatment with E2 for 48 h, it modified the amount of mRNA for BMP4, BMP receptors, and activin in ewe pituitary (also known as Acvr1) is able to induce BMP signaling (ten Dijke et al. 1994, Macías-Silva et al. 1998, Lee et al. 2007) and was previously shown to increase at the secondary FSH surge in ewe (Fafioffe et al. 2004). Nonetheless, BMP4 and BMP2 are known to bind to BMPR1A (ALK3) and BMPR1B (ALK6), whereas other BMPs bind more strongly to ALK2 (ten Dijke et al. 1994, Miyazono et al. 2010). In the murine gonadotrope cell line LBT2, the endogenous signal-propagating type I receptor at least for BMP2, structurally close to BMP4, would be BMPR1A (Ho & Bernard 2009). BMPs also show promiscuity in their binding to type II receptor. For example, in the absence of BMPR2, BMP2 and BMP4 can use ActR2A (Yu et al. 2005). However, BMPR2 is present on the gonadotrope cells. Moreover, the level of ActR2A mRNA is similar throughout the estrous cycle (Fafioffe et al. 2004). All together, these data led us to study preferentially BMPR2, BMPR1A, and BMPR1B. The lack of variations in the expression of BMP4 and BMP receptor mRNA does not necessarily mean that pituitary BMP4 activity does not change. For example, in the rat, neither activin βB nor activin receptor mRNA expression changes at the time of the secondary FSH surge. Instead, the expression of follistatin, an activin binding protein, is modulated freeing up activins to stimulate FSH (Halvorsen et al. 1994). Hence, we followed the mRNA encoding noggin, a BMP4 antagonist. No change for the level of this mRNA was observed between the studied stages. We can argue that antagonists other than noggin can be involved. However, the absence of variation in the mRNA encoding Id2 (Peng et al. 2004), one BMP target gene reflecting the bioactivity of BMP4 in ewe pituitary cells, does not fit with this idea. All together, the data suggest that pituitary BMP4 activity did not significantly vary over the estrous cycle.

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Another factor of the same TGF-β family, activin, is considered a major intrapituitary regulator (Corrigan et al. 1991, Baratta et al. 2001, Padmanabhan & McNeilly 2001). To compare the expression patterns of both BMP4 and activin, we analyzed the mRNA levels of activin subunits. Surprisingly, no study about the expression of pituitary activin across the ovine estrous cycle is available. Interestingly, our results showed that the level of the mRNA for activin ββ-subunit is higher before the secondary FSH surge compared to the other studied stages of the cycle. Also, the percentage of pituitary cells immunodetected with antibodies raised against ββ-subunit increased before the secondary FSH surge. In contrast, neither the levels of the mRNA for inhibin α- and activin βA-subunits nor the percentage of pituitary cells immunodetected with anti-α antibody varied significantly across the cycle. Together with the high levels of ActR2, ALK2, and ALK4 mRNA observed during the secondary surge of FSH (Fafioffe et al. 2004), these results
BMP4, BMP receptors, and activin in ewe pituitary · C SALLO and others

reinforce the role of pituitary activin B as a major stimulus of FSH synthesis. Further evidence comes from exposure of ewe pituitary cells in culture to SB431542, an ALK4/5/7 inhibitor, showing inhibition of FSH release in a dose-dependent manner. These data contrast with the lack of changes in the BMP4 system and underline the difference between pituitary BMP4 and activin.

In cultured ewe pituitary cells, treatment of cells with GnRH for 6 h did not modify the level of activin βB mRNA. Similarly, in rat pituitary cells, chronic GnRH or rapid pulse frequency of GnRH did not affect activin βB mRNA levels in contrast to low pulse frequency (Demura et al. 1996, Dalkin et al. 1999). Treatment of pituitary cells with E2 for 48 h increased the level of activin βB mRNA. This result is in contradiction with the data obtained by Baratta et al. (2001) who reported a decrease in activin βB mRNA after E2 treatment. Both studies used ewe pituitaries collected during the non-breeding season, and the reasons for this discrepancy are unclear. They may rely on differences in experimental conditions such as incubation medium or the duration of E2 treatment. The fact that E2 increases activin βB mRNA is surprising and does not fit with the negative effect of E2 on FSHβ mRNA and FSH release. However, the increase, although reproducible, is weak and does not necessarily lead to a rise in activin protein. Moreover, we did not observe any difference in the amount of activin βB mRNA in vivo between luteal and late follicular phases when plasma concentrations of E2 increase. However, the percentage of immunoreactive cells for βB is decreased suggesting that E2 can affect post-transcriptional events. Further study at the protein level is required. Furthermore, factors other than E2 can contribute to the control of activin βB expression. For instance, the rise in activin βB mRNA occurring before the secondary surge of FSH could be attributed to several factors such as the drop in circulating inhibin concomitantly to plasma E2 decrease and/or to the low pulse frequency of GnRH.

Collectively, our data showed that changes in FSHβ expression were not associated with variations in the expression of BMP4, BMP receptors, Id2, or noggin. The constant levels may reflect a constitutive BMP signaling in the pituitary. Nonetheless, treatment of pituitary cells with noggin did not modify Id2 level, at least in vitro, suggesting the absence of endogenous signaling. Moreover, blocking the action of BMP4 potentially produced by pituitary cells with noggin does not affect the in vitro release of FSH. Hence, the results do not favor the hypothesis that local BMP4 would play a regulatory role on FSH secretion during the estrous cycle. However, we cannot exclude the possibility that other BMPs can affect FSH production. Previous studies demonstrated that pituitary exhibits at least BMP2 and BMP7 mRNA expression (Huang et al. 2001, Faure et al. 2005). Alternatively, one can argue that BMP receptors, and potentially BMP4, are widely expressed in the pituitary making it possible that changes in their expression in the gonadotrope cells are masked by stable expression in other cells. However, we previously reported that the vast majority of cells bearing the BMPR1A and BMPR2 was identified as gonadotropes (Faure et al. 2005). To confirm the lack of variations, it will be interesting to examine the expression of mRNAs for BMP4, BMP receptors, or Id2 at the gonadotrope cell level using in situ hybridization or immunohistochemistry. In addition, minor changes in mRNA expression evading detection may be sufficient to exert a paracrine/autocrine action. Nevertheless, the fact that treatment of pituitary cells with noggin, an antagonist of BMP4 and also BMP2 and BMP7, did not modify FSH release does not support this idea. This does not rule out a role for BMP4 on FSH synthesis and release in physiological conditions other than the estrous cycle such as pituitary development and/or in pituitary tumor. In contrast to BMP4, the amount of activin βB mRNA as well as the βB-subunit in the pituitary was shown to increase before the secondary FSH surge strongly suggesting that activin is a major stimulus of FSH synthesis necessary for the secondary FSH surge. Moreover, blocking the action of activin potentially produced by pituitary cells with SB431542 in vitro inhibited FSH release demonstrating the importance of local activin. Further study is now needed to better identify the regulatory factors of activin synthesis related to FSH secretion.

In conclusion, this study expands current knowledge on local BMP4 and activin actions in the regulation of FSH synthesis and release. Our study presents a line of data that are not in favor of a paracrine/autocrine action of BMP4, in contrast to activin, on FSH production in adult pituitary during the estrous cycle. However, BMP4 or other BMPs may play a role as endocrine factors. Indeed, Herrera & Inman (2009) showed the presence of BMP4, BMP6, and BMP9 in bovine serum. Further experiments are required to determine the presence or absence of BMPs in ovine serum and their physiological involvement in the regulation of FSH synthesis and release.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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