BMP-4 inhibits follicle-stimulating hormone secretion in ewe pituitary

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Abstract

Activins and inhibins, members of the transforming growth factor-beta family are able to stimulate and inhibit, respectively, FSH synthesis and release. Other members of this superfamily, the bone morphogenetic proteins (BMPs), may also affect FSH synthesis in the mouse. The aim of this work was to determine whether BMPs are expressed in the ovine pituitary and whether they play a role in the regulation of FSH release.

The mRNAs encoding BMP-2, BMP-4, BMP-7 and the oocyte-derived growth factor, growth differentiation factor (GDF)-9 were detected in the pituitaries of cyclic ewes by reverse-transcriptase PCR, as well as the mRNAs encoding the BMP type I receptors, BMPR-IA (activin-receptor-like kinase (ALK)-3) and BMPR-IB (ALK-6), and type II receptors (BMPR-II). Immunolabeling of pituitary sections revealed the presence of BMPR-IA (ALK-3) and BMPR-II in gonadotrope cells. To investigate the potential effects of BMPs on FSH secretion, ewe

pituitary cell cultures were treated with BMP-4 (10^{-11} M to 10^{-9} M) for 48 h. Interestingly, FSH release was decreased in a dose-dependent manner. At 10^{-9} M BMP-4 both FSH concentration and FSH β mRNA expression were reduced by 40% of control values. In contrast, there was no inhibitory effect on either LH or LH β mRNA expression. A similar result was found with BMP-6. BMP-4 triggered the phosphorylation of Smad1, suggesting that the effect of BMP-4 on FSH secretion is due to the activation of the BMPs signaling pathway. Furthermore, BMP-4 blocked the stimulatory effect of activin on both FSH release and FSH β mRNA and amplified the suppression of FSH release and FSH β mRNA levels induced by 17 β -estradiol. These results indicate that a functional BMP system operates within the sheep pituitary, at least *in vitro*, to decrease FSH release and to modulate the effect of activin.

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Introduction

In females the gonadotropins FSH and LH produced by pituitary gonadotropes are required for terminal follicular growth and regulation of ovulation number (McNeilly *et al.* 1992). The patterns of the synthesis and release of LH and FSH diverge in several physiological situations. However, the mechanisms involved in LH and FSH differential regulation are still unclear. Gonadotropin synthesis and release are orchestrated by an interplay of hypothalamic, gonadal and pituitary factors. The primary stimulus for synthesis and release of FSH and LH appears to be the hypothalamic gonadotropin-releasing hormone (GnRH). Nevertheless, depending on pulse frequency or amplitude, GnRH exerts a differential effect on both hormones. While a high frequency of GnRH pulses is favourable to LH secretion, a low frequency is associated with the production of FSH (Dalkin *et al.* 1989, Molter-Gerard *et al.* 1999). Other factors such as gonadal steroids, progesterone and estrogens can also differentially modulate

the secretion of LH and FSH (Mercer *et al.* 1993, Molter-Gerard *et al.* 2000). In addition to GnRH and steroids, some members of the transforming growth factor-beta (TGF- β) superfamily, particularly activins and inhibins, are involved in specific regulation of FSH production. Activin is a dimer of two β subunits (A and B). The association of the subunits results in activin A, activin B or activin AB, each of which increases FSH β mRNA expression and FSH release (Carroll *et al.* 1989). Activins are produced by the gonads and the pituitary where they act as paracrine factors (Knight 1996, Bilezikjian *et al.* 2001, Welt *et al.* 2002, Lin *et al.* 2003). Inhibin is a dimer of an α subunit and either β A or β B subunits forming inhibin A or B respectively, each of which suppresses FSH synthesis and release (De Kretser & Robertson 1989). They are produced mainly in the gonads and act through an endocrine pathway. Recently other molecules of the TGF- β superfamily, the bone morphogenetic proteins (BMPs) were shown to play a role in FSH regulation (Huang *et al.* 2001, Otsuka & Shimasaki 2002).

Initially, BMPs were identified through their actions on bone morphogenesis. They are now known to have effects on growth, differentiation, apoptosis and other phenomena in many cell types (for review, Shimasaki *et al.* 2004). BMP signaling occurs through a heteromeric complex with a type BMPR-IA (ALK-3) or BMPR-IB (ALK-6) and type BMPR-II serine/threonine kinase receptors (Kawabata *et al.* 1998, Miyazono *et al.* 2001). The ligand binding induces the trans-phosphorylation of the type I receptor by the type II receptor. Consequently, the activated BMPR-I phosphorylates cytoplasmic proteins called receptor-activated Smads (R-Smads) which interact with a co-Smad, Smad4. BMP signaling recruits the specific R-Smads, Smad1, Smad5 and Smad8 and the R-Smad-co-Smad complex translocates to the nucleus to promote specific gene expression (for review, Zwijsen *et al.* 2003).

It is now admitted that BMPs play a crucial role in reproduction. In sheep, natural mutations of the BMP system alter the ovulation rate. For instance, in the Inverdale, Hanna, Cambridge and Belclare phenotypes, mutations in the BMP-15 gene lead to hyperprolificity in heterozygous ewes, while homozygous ewes are sterile (Galloway *et al.* 2000). A mutation in GDF-9, associated with alterations in follicular development, has also been identified in Belclare and Cambridge sheep (Hanrahan *et al.* 2004). In the Booroola phenotype, hyperprolificity is associated with a mutation in the intracellular serine/threonine kinase domain of the BMP receptor IB (Mulsant *et al.* 2001, Souza *et al.* 2001, Wilson *et al.* 2001). Several BMPs have recently been implicated as autocrine/paracrine regulators of ovarian follicle development (Knight & Glistler 2003, Shimasaki *et al.* 2004 (review)). For instance, BMP-15 produced by oocytes stimulates granulosa cell proliferation (Otsuka *et al.* 2000). BMP-4 and BMP-7 expressed by theca cells affect follicle development (Lee *et al.* 2001) and enhance FSH action on granulosa cells by stimulating estradiol (Shimasaki *et al.* 1999, Souza *et al.* 2002, Glistler *et al.* 2004) and decreasing progesterone production (Shimasaki *et al.* 1999, Fabre *et al.* 2003). Besides the regulatory role of BMPs in the ovary, little is known about the action of BMPs at the pituitary level, particularly in species other than rodents.

In mouse embryos, the role of BMPs in pituitary organogenesis is well documented. BMP-4 is required for induction and formation of the Rathke's pouch rudiment (Takuma *et al.* 1998) while BMP-2 acting with FGF-8 in opposite gradients determines gonadotrope cell differentiation (Ericson *et al.* 1998, Dasen *et al.* 1999). In the adult mouse pituitary, high concentrations of BMP-6 and BMP-7 stimulate the activity of transfected ovine FSH β promoter and the release of FSH (Huang *et al.* 2001). In the adult rat pituitary, BMP-15 stimulates specifically FSH release (Otsuka & Shimasaki 2002). Taken together, these data suggest that BMPs may act as regulatory factors in the pituitary, at least in rodents.

To extend our knowledge of FSH synthesis regulation, we investigated the potential role of BMPs in the ewe pituitary. Our data showed that BMP receptors and BMP-2, BMP-4, BMP-7 and GDF-9 mRNAs were present in the pituitary. Interestingly, BMP-4 and BMP-6 inhibited the FSH, but not the LH release from primary pituitary cells. In comparison with other physiological factors, we found that BMPs antagonized the activin effect known for stimulating FSH release and amplified the inhibitory action of 17 β -estradiol. Thus, in the sheep pituitary BMP-4 and BMP-6 act to suppress FSH production.

Materials and Methods

Reagents

Cell culture reagents used were DMEM (Dulbecco's modified eagle's medium) and F12 (Nutrient mixture F-12 Ham) from Sigma (Saint Louis, MO, USA). Gentamicin, nistatine, L-ascorbic acid, apo-transferrin, FCS (fetal calf serum) and BSA (bovine serum albumin) were purchased from Sigma. Collagenase A and DNase I were from Roche Diagnostics Ltd (Meylan, France). Human recombinant Activin-A, BMP-6 and BMP-4 were obtained from R&D systems (Lille, France). 17- β estradiol was from Sigma. Rabbit antibodies against phosphorylated Smad1 and against Smad1 protein were from Upstate Biotechnology (Euromedex, Mundolsheim, France). Mouse monoclonal antibody directed against bovine LH β (bLH β 518B7) was obtained from J F Roser (Department of Animal Science, University of California, USA) (Matteri *et al.* 1987). Rabbit polyclonal antibody directed against BMPR-IA (amino acid 181–202; ten Dijke *et al.* 1994), BMPR-IB (amino acid 151–168; ten Dijke *et al.* 1994) and BMPR-II (amino acid 185–202; Rosenzweig *et al.* 1995) were kindly given by C H Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden).

Tissue collection

Pituitaries from Ile de France, Merinos d'Arles and Scottish Blackface ewes were collected throughout the year and dissociated for cell cultures or immersed in Bouin's Holland fixative containing HgCl₂ for immunohistochemistry. Some pituitaries and ovarian follicles were used for RNA extraction.

Reverse transcription-PCR on whole tissues

Total RNA from ewe pituitaries and ovarian follicles were extracted using TRI Reagent (Sigma). Complementary DNA was synthesized from 1 μ g RNA in a volume of 20 μ l containing 150 ng oligodT (Promega, Charbonnières, France),

Table 1 Oligonucleotide primer sequences used for PCR (A) and Real-time PCR (B).

A PCR

	Forward sequence	Reverse sequence	Annealing temperature (°C)	MgCl ₂ concentration (mM)	Product size (bp)
Primer					
BMP-2	gTCACAgATAAggCCATTgC	CCTCCACAACAATgTCCTgA	58	1.5	403
BMP-4	ggTCACCTTTggCCATgATg	ACCTTgTCATACTCATCCAg	58	2	359
BMP-6	AAGCAGCCCTTCATggTggC	CATgTgTgCgTTgAgAgggA	58	1	296
BMP-7	AgAgCATCAACCCCAAgTTg	CgATgATCCAgTCCTgCCAg	62	1	284
BMP-15	ggATCACTggATCATTgCTC	gCAATCATAACCCTCATACTC	58	1.5	250
GDF-9	TAgTCAGCTgAagtgggACA	ACgACAggTACACTTAgTgg	58	1	275
BMP-RIA	AACACATgCATAACTAATgg	ACCATCTgAATCTgTTTggC	54	1.5	493
BMP-RIB	AgATTggAAAAGgTCgCTATg	CgAgTgTTgggTggTATg	54	1.5	486
BMP-RII	ATggAACATgACAACATTgC	CCTgggAAGgTCTgTACA	54	1.5	523

B Real time PCR

	Forward sequence	Reverse sequence	Probe
Primer			
FSHβ	CACgAgggTgCTTTTCCATT	TgggACCgTATCCCTAgCTTT	AATAATCTTAgAAATCCTCTCAggCAATCCCTTCCTC
LHβ	CCgCTCCCAgATATCCTCTTC	TgCTggCTTTgggAgTTAgg	AAggATgCCCCACTTCAATCTCCCAT

1 mM dNTPs, 20 U of RNasin, 1 × RT PCR buffer [from 5 × assay buffer B (250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM MgCl₂, 50 mM DTT)], 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 reverse transcription at 37 °C for 1 h. PCR was carried out in a volume of 25 µl containing 2.5 µl RT reaction mixture, 1 × PCR buffer [from 10 × PCR buffer without MgCl₂ (500 mM KCl, 100 mM Tris HCl (pH 9) and 1.0% Triton X-100), 50 pmol of each primer, indicated concentrations of MgCl₂, 0.2 mM dNTPs and 10 U *Taq* polymerase (Promega). PCR reactions were performed for 30 cycles of 30 s at 94 °C, 2 min at indicated annealing temperature, and 3 min at 72 °C. For primer sequences and details, see Table 1A.

Immunohistochemistry

After fixation and dehydration, sheep pituitaries were embedded in paraffin wax. Sections (5 µm thickness) were dewaxed and re-hydrated in xylene then in decreasing concentrations of alcohol (100, 90 and 75%). Antigen retrieval was performed by steaming the sections in a pressure cooker in citrate buffer (0.01 M) pH 6.0 for 5 min, then cooled down for 20 min. After two 5 min washes in PBS a combined avidin-biotin block was performed according to the manufacturer's instructions (Vector Laboratories, Peterborough, UK). Sections were incubated at 4 °C overnight with the monoclonal anti-LHβ diluted 1:250 in PBS containing 20% normal goat serum and 5% BSA (PBS/NGS/BSA). Negative controls

were performed by replacing the first antibody with normal goat serum. After washes in PBS-Tween 20 (0.05%; PBST) and PBS, slides were incubated in goat anti-mouse Alexa 488 (Molecular Probes, Eugene, OR, USA) and diluted at 1:200 in PBS for 1 h. After washes in PBS, slides were blocked in PBS/NGS/BSA for 30 min. Sections were then incubated at 4 °C overnight with rabbit polyclonal antibody directed against BMPR-IA, BMPR-IB or BMPR-II diluted in PBS/NGS/BSA at 1:100, 1:100 and 1:25, respectively. Negative controls were performed by replacing either BMPR antibody with normal goat serum. After washes in PBST and PBS, slides were incubated with goat anti-rabbit biotinylated antibody (Dako, Cambridge, UK) and diluted 1:500 for 30 min. After three 5 min washes in PBS, sections were incubated with streptavidin Alexa 546 (Molecular Probes) diluted at 1:200 in PBS/NGS/BSA and counterstained for 2 min with propidium iodide at 1:2000 for 2 min. Sections from sheep ovaries were performed as positive controls (Souza *et al.* 2002). The slides were examined using LSN 510 meta confocal microscope.

Sheep pituitary cell cultures

Pituitaries were finely sliced and placed in F12 supplemented (3 µg/ml gentamicin, 2 µg/ml nistatine, 5% FCS, 0.4 mg/ml collagenase A and 0.025 mg/ml DNase I) and incubated for 1 h30 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 resuspended in culture medium (DMEM supplemented with 3 µg/ml gentamicin, 2 µg/ml nistatine,

100 μ M L-ascorbic acid, 5 μ g/ml apo-transferrin and 5% FCS). Cells were plated in 48-well plates at 200 000 cells/well, 12-well plates at 1×10^6 cells/well or 6-well plates at 2×10^6 cells/well in 500 μ l, 1 ml or 2 ml respectively of culture medium (DMEM with 5% FCS), and allowed to attach for 2 days in a humidified atmosphere with 5% CO₂ in air at 37 °C. Media were replaced with serum-free DMEM containing 0.1% BSA. One hour later, the medium was changed and DMEM–0.1% BSA alone (control) or supplemented with test substances at different concentrations (see Results) was added. Media were collected 48 h later to assay for FSH and LH.

LH and FSH assays

The concentrations of FSH and LH were determined using double antibody ELISA immunoassays for all experiments, except for the estradiol experiments. For these latter, performed in Scotland, radioimmunological assay (RIA) for FSH was used.

ELISA LH and FSH were assayed in duplicate aliquots of pituitary cell culture supernatants. For LH, microtitration plates (Maxisorp C96, Nunc, France) were coated overnight at 4 °C with 100 μ l/well of a monoclonal antibody to bovine LH β -subunit 518B7 (Matteri *et al.* 1987) and diluted 1:3200 in carbonate buffer. Plates were then washed and blocked with PBS pH 7.4 containing 0.09% Tween 20 and 12.5% Sea-Block (Pierce, Brebières, France). Purified ovine LH (oLH CY1083) used as standard, controls and supernatants were added at 20 μ l/well along with 80 μ l/well of dilution medium (PBS containing 0.09% Tween 20, 12.5% Sea-Block and 2% normal rat serum). Plates were incubated overnight at 4 °C. After removal of unbound material, biotinylated monoclonal antibody to human α -subunit (Dirnhofer *et al.* 1994) diluted at 23 ng/100 μ l/well in PBS containing 0.1% Tween 20 and 1% rat serum was added for 1 h at 37 °C. Plates were washed and horseradish peroxidase-labelled neutravidin (Pierce) was added at 25 ng/100 μ l/well. After 25 min at room temperature in obscurity and washing, peroxidase activity was developed with 100 μ l tetramethylbenzidine/well (Dako) for 30 min at room temperature. The reaction was stopped with 50 μ l HCl/H₂SO₄/H₂O (1 v/1 v/6 v) per well and the signal was measured at 450 nm and 620 nm. The minimum detectable concentration for LH was 0.1 ng/ml. The intra- and inter-assay coefficients of variation of the control averaged 4% and 10%, respectively. The cross-reaction with oFSH was 0.01%.

For FSH, concentrations were measured as described for LH assay with the following modifications. Plates were coated overnight at 4 °C with 100 μ l/well of a monoclonal antibody to ovine FSH β subunit (Henderson *et al.* 1995) and diluted 1:100 in carbonate buffer. Purified ovine FSH (NIH RP2) used as standard, controls and supernatants

were added at 50 μ l/well along with 50 μ l/well of dilution medium. The minimum detectable concentration for FSH was 0.2 ng/ml. The intra- and inter-assay coefficients of variation of the control were 2.6% and 2.1%, respectively. The cross-reaction with oLH was 0.07%.

RIA RIA assay on ovine FSH was previously described (McNeilly *et al.* 1976, 1986). The sensitivity of the FSH RIA was 0.1 ng/ml and the intra- and inter-assay coefficients of variation were less than 10%.

Quantitative RT-PCR from pituitary cell cultures

Ovine FSH β and LH β primers and probe were designed using Primer Express software (PE Biosystems, Warrington, Cheshire, UK) and synthesized by PE Biosystems (Table 1B). Ribosomal 18S primers and probe were from a Taqman RNA Control Reagents kit (VIC labeled probe; PE Biosystem).

Total RNA from 12-well plates were extracted using the Agilent Total RNA Isolation Mini Kit. RNA concentration was measured using RNA 6000 Nano Assay (Agilent Technologies, Stockport, UK). Aliquots of total RNA were treated with DNase I using DNA-free (Ambion Ltd, Huntingdon, Cambs, UK) according to manufacturer's protocol. Samples were then reverse transcribed, using oligod(T)16, with Taqman Reverse Transcription Reagents (PE Biosystems) according to the manufacturer's protocol, using a program of 10 min at 25 °C, 30 min at 48 °C and 5 min at 95 °C. DNase-treated RNA was approximatively added at 25–50 ng/ μ l. For PCR, a reaction mix was prepared consisting of Taqman Universal PCR Master Mix (1 $\times$), FSH β or LH β forward and reverse primers (300 nM each), FSH β or LH β probe (200 nM), ribosomal 18S forward and reverse primers (50 nM each) and ribosomal 18S probe (200 nM). This was aliquoted for each sample in separated tubes, then cDNA was added at 1 μ l/25 μ l reaction mix (equivalent to 25–50 ng total RNA/25 μ l). Aliquots of 25 μ l were dispatched into a 96-well PCR plate, with each sample added in duplicate, optical caps fixed onto the plates and the PCR reaction run on an ABI Prism 7900 PCR machine (PE Biosystems) using standard conditions. Controls including cDNA prepared without Multiscribe reverse transcriptase enzyme were done to check for efficiency of DNase treatment. Prior to analysis, a validation assay was performed to demonstrate that amplification of FSH β gene and the reference (18S) were approximately equal, whereas the amplification of LH β gene and 18S were not equal. FSH β and LH β mRNA quantifications were performed by the $\Delta\Delta$ Ct method and the standard curve method respectively (Bulletin number 2; PE Biosystems). A standard PCR reaction using the FSH β and the LH β primers was run on an agarose gel to confirm amplification of a single product of the correct size.

Western blotting analysis of Smad proteins

To investigate Smad-1 activation by BMP-4, ewe pituitary cells were cultured at 2×10^6 cells/well in 6-well plates for 48 h. Media were changed to remove non-attached cells and replaced by fresh media containing serum-free DMEM-0.1% BSA alone or supplemented with 10^{-9} M (50 ng/ml) of BMP-4 for 30, 60 and 90 min. Cells were then washed once on ice with cold PBS and lysed in lysis buffer (150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% Igepal CA-630, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 10 mM sodium orthovanadate, proteases inhibitor cocktail). The concentrations of the proteins were determined with BC Assay Protein Quantitation kit (Interchim, Montluçon, France). After 1 min at 95 °C in the reducing SDS-PAGE buffer containing β -mercaptoethanol, 15 μ g proteins were separated in 10% SDS-PAGE gels and electroblotted onto Immobilon-P membranes (Millipore corporation, Bedford, MA, USA). Membranes were blocked for 1 h at room temperature in Tris-buffered saline-0.1% Tween containing 5% fat-free dry milk and incubated with the anti-phospho Smad1 or the anti-Smad1 protein diluted at 1:4000 and 1:1000, respectively, overnight at 4 °C. After washing, the membranes were incubated with the secondary antibody, a peroxidase-conjugated anti-IgG (Biorad, Marnes la Coquette, France) diluted 1:10000 for 1 h. Immunoreactive proteins were detected using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Orsay, France). Membranes were exposed to Hyperfilm ECL (Amersham Pharmacia Biotech) for 5 min.

Statistical analysis

Results are reported as mean $\pm$ S.E.M. All experiments were performed in triplicate in at least three different experiments. For each figure, the number of experiments shown is indicated in the legend. The effects of increasing doses of BMP-4 on FSH concentrations were analysed by one-way ANOVA followed by Newman-Keuls post-test. The time course experiment was analysed by two-way ANOVA followed by Bonferroni post-test in order to appreciate the 'time effect' as well as the 'ligand' effect. Comparison between BMP-4 or BMP-6 and control groups on FSH and LH hormone concentrations and mRNA expression levels were analysed by an unpaired *t*-test. Comparison between BMP-4, activin, BMP-4 plus activin, estradiol, BMP-4 plus estradiol and control groups on FSH hormone concentrations and FSH β mRNA expression levels were analysed by one-way ANOVA followed by Newman-Keuls post-test. $P < 0.05$ was considered significant. The statistical analysis was performed using the GraphPad Prism version 4.00 program for Windows (GraphPad Software, San Diego, CA, USA: www.graphpad.com).

Results

BMPs and BMP receptors are expressed in the ewe pituitary

To test whether ewe pituitary cells express BMP receptors and BMPs, RT-PCR was performed on Ile de France and Merinos d'Arles ewe pituitaries using primers described in Table 1A. After 30 cycles, DNA fragments were detected at expected sizes for BMP-2, BMP-4, BMP-7, GDF-9, BMP-RIA (ALK-3), BMP-RIB (ALK-6) and BMP-RII (Fig. 1A, B). No PCR product was detected for BMP-6 and BMP-15 in the pituitary. Positive controls were done on sheep ovarian follicles. In follicles, DNA fragments were observed for BMP-6 and BMP-15 (Fig. 1C, D). When negative controls were performed by omitting reverse transcriptase in the RT reaction, no PCR products were detected (not shown). Sequencing of all fragments confirmed product identity.

BMP receptors IA and II are present in gonadotrope cells

Immunohistochemistry analysis revealed BMPR-IA, BMPR-IB and BMPR-II positive cells in the pituitary. The vast majority of cells bearing the BMPR-IA and BMPR-II are identified as gonadotropes by colocalization of BMPR-IA or BMPR-II and LH immunoreactivity (Fig. 2A, C). In contrast, BMPR-IB did not colocalize with LH (Fig. 2B).

BMP-4 and BMP-6 inhibit FSH secretion and mRNA levels in the ewe pituitary

To determine whether BMPs were capable of modifying gonadotropin release from the ewe pituitary, cells were incubated with BMP-4. A dose-response study showed that treatment of cells with BMP-4 for 48 h inhibited FSH secretion from pituitary cells in a dose-dependent manner (Fig. 3A). The maximal inhibition of BMP-4 on FSH release was obtained with concentrations from 5×10^{-10} M (25 ng/ml). A time course experiment showed that significant effect with BMP-4 at 10^{-9} M (50 ng/ml) was observed from 24 h (Fig. 3B). When 10^{-9} M BMP-4 were used to treat cells for 48 h in additional experiments, we confirmed that BMP-4 inhibited FSH release by 40% ($P < 0.01$) compared with control (Fig. 4A). LH release was not decreased (Fig. 4B). Similarly, FSH β mRNA expression was decreased by 30–40% ($P < 0.05$) but not LH β mRNA expression (Fig. 4C, D). When cells were treated with 2×10^{-9} M (100 ng/ml) BMP-6 for 48 h, a similar inhibitory effect on FSH release was observed (Fig. 4E, F). These inhibitory effects of BMPs were found for Ile de France, Merinos d'Arles and Scottish Blackface ewes.

BMP-4 stimulates the BMP signaling pathway

To determine whether BMP-4 could activate the BMP signaling pathway, proteins were extracted after 30, 60 or

Figure 1 Expression of BMP ligands and receptors in sheep pituitary and ovarian follicles. Total RNA was extracted from sheep pituitaries (A,B) and ovarian follicles (C,D). The mRNAs encoding BMP ligands (A,C) and receptors (B,D) were analysed by RT-PCR at 30 cycles. DNA fragments were electrophoresed in 1% agarose gel and visualized by ethidium bromide staining.

90 min treatment with 10^{-9} M BMP-4. The activation of Smad-1 was followed by western blot analysis using an antibody which recognizes the dual serine phosphorylated Smad-1 (Ser 463/465) with an apparent molecular weight of 65–66 kDa in the phosphorylated state. Figure 5 shows that BMP-4 induced the apparition of a 66 kDa band within 30 min. The lower band observed on the blot corresponded to a non-specific band. In control samples harvested at the same time points, no phospho Smad1 immunoreactivity was detected. For Smad-1 protein, the intensity of the band did not change between control and BMP treatment (Fig. 5).

BMP-4 antagonises activin effects

To test whether BMP-4 was able to counteract the stimulatory effect of activin on FSH secretion and FSH β mRNA expression, cells were incubated with both 2×10^{-9} M activin (50 ng/ml) and 10^{-9} M BMP-4. BMP-4 antagonised the activin effect on FSH secretion (Fig. 6A) and on FSH β mRNA levels (Fig. 6B).

BMP-4 amplifies the effect of 17- β estradiol

To test whether BMP-4 was able to amplify the inhibitory action of estradiol on FSH production, the effects of simultaneous treatment with BMP-4 and 17- β estradiol were determined. A dose-response study showed that 17- β estradiol inhibited the FSH secretion from pituitary cells in a dose-dependent manner (Fig. 7A). At 10^{-12} M, 17- β estradiol inhibited by 30–40% FSH release ($P < 0.05$ vs control) and maximal inhibition (around 70%) was obtained with 10^{-11} M ($P < 0.001$ vs control; $P < 0.05$ vs 10^{-12} M). FSH β mRNA expression was decreased by 80% ($P < 0.001$ vs control) with 10^{-11} M 17- β estradiol

(Fig. 7B). When cells were incubated with both BMP-4 (10^{-9} M) and 17- β estradiol (10^{-12} M or 10^{-11} M), a stronger inhibition of FSH release was observed compared with either factor alone (Fig. 7A). This was also observed on FSH β mRNA levels when cells were treated with both BMP-4 (10^{-9} M) and 17- β estradiol (10^{-11} M) (Fig. 7B).

Discussion

The synthesis and the secretion of the gonadotropins are differentially regulated by GnRH, ovarian hormones and pituitary factors. Control of FSH production includes specific factors belonging to the TGF- β superfamily, activin and inhibin. Recent data in rodents emphasize the potential role of other members of the same family, the BMPs, in the regulation of FSH synthesis and release (Huang *et al.* 2001, Otsuka & Shimazaki 2002). We investigated here whether BMPs could participate to control FSH synthesis and secretion in the ewe. We first demonstrated that ewe pituitary does express a set of BMP mRNAs, BMP-2, BMP-4, BMP-7 and GDF-9. Furthermore, BMP type IA (ALK-3) and II receptors are present in the gonadotrope cells suggesting that a functional BMP system can act within the pituitary. Indeed, we observed that BMP-4 and BMP-6 decreased FSH β mRNA expression and FSH release from ewe pituitary cells, whereas similar inhibitory effects were not observed on LH secretion.

The presence of BMP-2, BMP-4 and BMP-7 mRNA in the adult ovine pituitary corroborates the results of Souza *et al.* (2003). Moreover, for the first time, GDF-9 mRNA, known to be specifically expressed in oocyte (Yan *et al.* 2001) was detected in the pituitary. However,

Figure 2 Immunohistochemical double staining for BMPR and LH. BMPR-IA, BMPR-IB and BMPR-II proteins were detected in the pituitary by immunohistochemistry. Ewe pituitary sections were incubated with mouse monoclonal antibody directed against LH (1:250) to detect gonadotrope cells followed by goat Alexa 488-conjugated anti-mouse IgG. In a second step, the same sections were incubated with rabbit polyclonal antibodies directed against BMPR-IA (1:100) (A), BMPR-IB (1:100) (B) or BMPR-II (1:25) (C) followed by goat anti-rabbit biotinylated antibody and then Alexa 546 labelled streptavidin. Nuclei were counterstained with propidium iodide. Most BMPR-IA and BMPR-II positive cells are also positive for LH. BMPR-IB positive cells are not positive for LH (B). Bar=10μm.

we did not detect BMP-6 and BMP-15 mRNA in ewe pituitary after 35 PCR cycles, whereas they are clearly observed in the ovarian follicles. In the mouse, these mRNAs appeared in the pituitary after more than 30–35 cycles (Huang *et al.* 2001, Otsuka & Shimazaki 2002). Hence, the pattern of BMP expression appears to be different among species. In the ewe, our results suggest that BMP-2, BMP-4, BMP-7 and GDF-9 could have important actions at the pituitary level. Because BMP-4

mRNA was strongly expressed in pituitary, we examined its effect on FSH β expression and FSH release in the ewe pituitary cells in primary culture. The effect of BMP-6 reported as an FSH stimulator in mouse pituitary by Huang *et al.* (2001) was also examined. The findings of inhibitory effects of both BMP-4 and BMP-6, at low concentrations, on FSH β mRNA expression and FSH release from ewe pituitary cells were unexpected. Indeed, similar doses of BMP-6 and BMP-7 had no effect on FSH

Figure 3 Dose response (A) and time course (B) of BMP-4 on FSH release from ewe pituitary cells. For the dose response, cells were cultured in serum-free medium for 48 h in the presence of indicated concentrations of BMP-4. For the time course, cells were cultured in serum-free medium in the presence or absence (control) of 10^{-9} M of BMP-4 for indicated times. Media were collected and the concentrations of FSH were determined by ELISA. Values are the mean $\pm$ S.E.M. from one representative experiment with triplicate determinations. Three experiments were performed with similar results. a vs b, $P < 0.05$; a vs c, $P < 0.0001$; b vs c, $P < 0.01$; *, $P < 0.05$ vs respective control.

synthesis and secretion in mouse pituitary cells or the gonadotrope cell line, L β T2 (Yamashita *et al.* 1995, Huang *et al.* 2001, Otsuka & Shimasaki 2002) while a much higher concentration ($1 \mu\text{g/ml}$, i.e. 2×10^{-8} M) increased FSH transcription and secretion. Another BMP, BMP-15 exhibited a stimulatory effect in rodents (Otsuka & Shimasaki 2002). The inhibitory effect of BMP-4 on ovine FSH production was not accompanied with an effect on pituitary cell proliferation as judged by the absence of changes in pituitary cell number after BMP treatment (data not shown). In mouse experiments, it has

been suggested that BMP-6 and BMP-7 could transduce an activin-like signal since certain BMPs can bind to activin type II receptor present on mouse gonadotropes (Yamashita *et al.* 1995). Moreover, follistatin, known to neutralize activin, has been observed to bind several BMPs (Iemura *et al.* 1998) and local concentrations of follistatin may be sufficient to complex with BMP and compete with activin. However, in the ewe pituitary BMPs do not exert an activin-like signal although, activin type II and type I (ALK-2 and ALK-4) receptors are expressed (Dupont *et al.* 2003) as well as follistatin (Farnworth *et al.* 1995). Rather, BMPs can stimulate their own receptors. Activation of BMP type I receptors (ALK-3 and ALK-6) is known to induce the phosphorylation of intracellular receptor-specific Smad proteins (R-Smad), Smad1, Smad5 and Smad8 (Miyazono *et al.* 2001). In ewe pituitary cells, we observed that phosphorylation of Smad1 occurred within 30 min of BMP addition. This result indicates that BMPs induce their own signaling pathway. Whether the activation of Smad1 is responsible for the decrease of FSH β mRNA levels and FSH release has yet to be confirmed.

Upon phosphorylation of the R-Smad proteins, these latter associate with a common partner, Smad-4 and translocate to the nucleus. The complexes R-Smad-Smad4 are then capable of binding DNA on a Smad binding element (SBE). The regulation of target gene requires additional interaction with transcription factors, either coactivators or corepressors (for review, Massague & Wotton 2000). Both SBEs and transcription factors may be cell type specific. For instance, a SBE was recently identified in the rat FSH β subunit gene that is required for full activin responsiveness (Suszko *et al.* 2003). Interestingly, whereas this site is conserved in the mouse gene, it is not present in the ovine, bovine, porcine or human genes, suggesting that important species-specific differences exist in activin regulation of FSH β gene expression. Similar differences probably exist in BMP regulation of target genes. Given that BMP-15 was found to stimulate the activity of ovine FSH β promoter transfected in mouse L β T2 gonadotrope cells, the presence of specific repressors rather than particular SBE in ovine gonadotropes could be favoured. Alternatively, because we did not determine whether BMPs act directly on FSH β promoter, we cannot exclude the possibility that BMPs affect FSH production via another target gene in gonadotropes or other cell type. Further experiments will help to discern between these hypotheses.

Considering the inhibitory effect of BMPs on FSH secretion from ewe pituitary cells, we questioned whether BMPs can affect the stimulatory effect of activin, an essential regulator of FSH. Activin is produced by the pituitary and exerts a paracrine action on FSH secretion (for review, Padmanabhan & McNeilly 2001). In the ewe, activin β B-subunit and activin receptors are expressed by the gonadotropes themselves (Dupont *et al.* 2003,

I. BMP-4

II. BMP-6

Figure 4 (I) Effect of BMP-4 on FSH (A) and LH (B) release and on FSH β (C) and LH β (D) mRNA expression from ewe pituitary cells. Cells were cultured in serum-free medium for 48 h with 10^{-9} M of BMP-4. Media were collected and the concentrations of FSH and LH were determined by ELISA. Values are the mean $\pm$ S.E.M. from ten experiments. a vs b, $P < 0.01$. Cells were harvested and total mRNAs were extracted. A real time PCR was performed to detect the relative level of FSH β or LH β mRNA expression compared with the 18S mRNA. Values are the mean $\pm$ S.E.M. from one representative experiment with triplicate determinations. Three experiments were performed with similar results. a vs b, $P < 0.05$. (II) Effect of BMP-6 on FSH (E) and LH (F) release from ewe pituitary cells. Cells were cultured in serum-free medium for 48 h with $2 \cdot 10^{-9}$ M of BMP-6. Media were collected and the concentrations of FSH and LH were determined by ELISA. Values are the mean $\pm$ S.E.M. from five experiments. a vs b, $P < 0.05$.

McNeilly *et al.* 2003) and the production of activin was suggested by the findings of an activin bioactivity from pituitary cell conditioned media (F Mathoux, D J Phillips and C Taragnat, unpublished observations). The action of activin is modulated by antagonists, mainly follistatin and inhibin which abolish the effect of activin.

In our study, BMP-4 and BMP-6 (not shown) were found to antagonize the activin effect, suggesting that they can participate with other activin antagonists to regulate FSH synthesis and secretion. The precise mechanisms by which BMP and activin interact are under investigation.

Figure 5 BMP-4 effect on Smad-1 phosphorylation. Pituitary cells were cultured in serum-free medium in presence or absence (control) of 10^{-9} M BMP-4 for indicated times. Total proteins (15 µg) were separated on 10% SDS-PAGE and transferred on PVDF membrane. Blots were probed with phospho-Smad1 antibody (upper) or with a Smad1 antibody (lower) and bands were revealed by ECL. ns refers to non specific.

Besides pituitary factors, another main regulator of FSH is estradiol. Estrogen receptors are expressed in gonadotropes in sheep (Sheng *et al.* 1998, Tobin *et al.* 2001) and injections of 17- β estradiol in ewes reduced FSH β mRNA concentrations and FSH release directly at the pituitary level (Mercer *et al.* 1993, Phillips *et al.* 1998, Molter-Gérard *et al.* 2000). Moreover, the treatment of ovine pituitary cells with 17- β estradiol showed a direct negative effect on FSH promoter (Miller & Miller 1996). In our study, estradiol also exerted a strong inhibition on FSH β mRNA expression and FSH release. Moreover, BMP-4 amplified this effect of estradiol. In other tissues, it was shown that estrogens and BMPs could interact through overlapping intracellular signaling mechanisms (Yamamoto *et al.* 2002). In pituitary prolactinomas in rodent and human where BMP-4 as well as 17- β estradiol induces cell proliferation, an additive effect of both BMP-4 and 17- β estradiol was observed (Paez-Pereda *et al.* 2003). In that model, Smad1 and Smad4 physically interacted with the estrogen receptor under BMP-4 stimulation (Paez-Pereda *et al.* 2003, Wu *et al.* 2003). A similar mechanism can occur in gonadotrope cells.

Whether BMP-4 exerts an inhibitory effect on FSH synthesis and release *in vivo* is not known at this date. In sheep, mutations have been identified either in BMPR-IB (ALK-6) in Booroola ewes (Mulsant *et al.* 2001, Souza *et al.* 2001, Wilson *et al.* 2001) or in BMP-15 in Inverdale and Hanna ewes (Galloway *et al.* 2000). Homozygous mutants for BMPR-IB and heterozygous mutants for BMP-15 exhibit a greater ovulation rate than wild type animals. Whether these mutants present changes in plasma FSH concentrations is a matter of controversy (McNatty *et al.* 1987, McNatty *et al.* 1991). However, we did not detect BMPR-IB in the gonadotrope cells. Hence, it is not surprising that the mutation does not significantly affect FSH release. We did not detect BMP-15 mRNA in the

Figure 6 Interaction between BMP-4 and activin effects on FSH release (A) and FSH β mRNA expression (B) in ewe pituitary cells. Cells were cultured in serum-free medium for 48 h in presence of 10^{-9} M BMP-4 and/or 2×10^{-9} M activin. Media were collected and the concentrations of FSH were determined by ELISA. Cells were harvested and total mRNAs were extracted. A real time PCR was performed to detect the relative level of FSH β mRNA expression compared with the 18S mRNA (B). Values are the mean $\pm$ S.E.M. from one representative experiment with triplicate determinations. Three experiments were performed with similar results. Bars with different letters indicate that group means are significantly different at $P < 0.05$.

pituitary, excluding the effect of the mutation at the pituitary level. Therefore, these mutation models are not suitable for understanding further the role of BMP-4 in FSH control.

In conclusion, BMP-4 and BMP-6 were shown to decrease FSH β mRNA expression and FSH release from ewe pituitary cells and are able to antagonize the effects of activin, at least *in vitro*. These effects were not found on LH secretion. Moreover, the detection of BMP-2, BMP-4, BMP-7 and GDF-9 mRNAs in the pituitary as well as the colocalisation of both types of BMP receptors on gonadotrope cells suggest that these BMPs can exert

Figure 7 Effect of 17- β estradiol in presence of BMP-4 on FSH release (A) and FSH β mRNA expression (B) in ewe pituitary cells. Cells were cultured in serum-free medium for 48 h in the presence of indicated concentrations of 17- β estradiol with or without 10^{-9} M of BMP-4. Media were collected and the concentrations of FSH were determined by RIA (A). Cells were harvested and total mRNAs were extracted. A real time PCR was performed to detect the relative level of FSH β mRNA expression compared with the 18S mRNA (B). Values are the mean $\pm$ S.E.M. from one representative experiment with triplicate determinations and three experiments were performed with similar results. Different letters indicate that group means are significantly different at $P < 0.05$. *, $P < 0.05$ vs 17- β estradiol or BMP individually.

paracrine actions on FSH production modulating activin and/or estradiol action. Therefore, the findings of a functional BMP system in the ewe pituitary emphasize the

role of BMPs in FSH control. Further experiments are required to establish the physiological importance of BMPs within the pituitary in the overall regulation of FSH synthesis and release.

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