Involvement of bone morphogenetic protein-4 in GH regulation by octreotide and bromocriptine in rat pituitary GH3 cells

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Abstract

Here we investigated roles of the pituitary bone morphogenetic protein (BMP) system in modulating GH production regulated by a somatostatin analog, octreotide (OCT) and a dopamine agonist, bromocriptine (BRC) in rat pituitary somatolactotrope tumor GH3 cells. The GH3 cells were found to express BMP ligands, including BMP-4 and BMP-6; BMP type-1 and type-2 receptors (except the type-1 receptor, activin receptor-like kinase (ALK)-6); and Smad signaling molecules. Forskolin stimulated GH production in accordance with cAMP synthesis. BRC, but not OCT, suppressed forskolin-induced cAMP synthesis by GH3 cells. Individual treatment with OCT and BRC reduced forskolin-induced GH secretion. A low concentration (0.1 μ M) of OCT in combination with BRC (1–100 μ M) exhibited additive effects on reducing GH and cAMP production induced by forskolin. However, a high concentration (10 μ M) of OCT in combination with BRC failed to

suppress GH and cAMP production. BMP-4 specifically enhanced GH secretion and cAMP production induced by forskolin in GH3 cells. BRC, but not OCT, inhibited BMP-4-induced activation of Smad1,5,8 phosphorylation and Id-1 transcription and decreased ALK-3 expression. Of note, in the presence of a high concentration of OCT, the BRC effects suppressing BMP-4-Smad1,5,8 signaling were significantly impaired. In the presence of BMP-4, a high concentration of OCT also attenuated the BRC effects suppressing forskolin-induced GH and cAMP production. Collectively, a high concentration of OCT interferes with BRC effects by reducing cAMP production and suppressing BMP-4 signaling in GH3 cells. These findings may explain the mechanism of resistance of GH reduction to a combination therapy with OCT and BRC for GH-producing pituitary adenomas.

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Introduction

Acromegaly is caused by excessive growth hormone (GH) secretion from pituitary GH-secreting adenomas. Treatment of acromegaly patients often involves combining surgical, pharmacological, and radiotherapeutic approaches to control endocrine profiles and reduce mortality. Somatostatin analogs and dopamine agonists are utilized as principal medications for acromegaly patients before and/or after pituitary surgery.

It has been generally recognized that a combination therapy with somatostatin analogs and dopamine agonists is potentially effective to reduce GH overproduction in acromegaly patients who are resistant to single medication regimens (Lamberts *et al.* 1986, Chiodini *et al.* 1987, Wagenaar *et al.* 1991, Marzullo *et al.* 1999). The combination treatment is also effective in *in vitro* studies using cultured pituitary tumor cells of acromegaly patients (Lamberts *et al.* 1987). For instance, a regimen using low-dose octreotide (OCT) and bromocriptine (BRC) is reported to be as efficacious as

high-dose OCT, indicating usefulness and tolerability of combination therapy in acromegaly patients (Li *et al.* 2000). Increased bioavailability of BRC during the combination with OCT has been suggested as a possible mechanism to explain the efficacy of the combination treatments (Flogstad *et al.* 1994).

Since dopamine agonists inhibit GH hypersecretion in 20% of acromegalic patients (Jaffe & Barkan 1992), there is a basis to expect that a combination regimen with dopamine agonists and somatostatin analogs would be an effective treatment. However, the efficacy of such combined treatment has yet to be firmly established due to some conflicting data. It was reported that when OCT is administered in three daily injections the additive effect of BRC on GH and insulin-like growth factor-I suppression is only negligible (Fredstorp *et al.* 1994). The presence of some resistant cases to a combination therapy in patients who were initially treated with OCT subsequently treated with dopamine agonists has also been observed (Cozzi *et al.* 2004). Thus, the detailed cellular

mechanism by which combination therapy suppresses GH production needs to be elucidated in order to distinguish medication-sensitive patients from non-responsive patients to the combination therapy. To approach the cellular mechanism of GH reduction and the resistance to combination treatment with OCT and BRC, we focused on the bone morphogenetic protein (BMP) system in the pituitary, which has recently received attention as a critical factor in the pathogenesis of certain functioning pituitary adenomas.

BMPs, which belong to the transforming growth factor- β superfamily, were originally identified as the active components in bone extracts capable of inducing bone formation at ectopic sites. A variety of physiological BMP actions in many endocrine tissues including the ovary (Otsuka *et al.* 2000, 2001, Miyoshi *et al.* 2007), pituitary (Otsuka & Shimasaki 2002, Takeda *et al.* 2003, 2007), thyroid (Suzuki *et al.* 2005), and adrenal (Suzuki *et al.* 2004, Kano *et al.* 2005, Inagaki *et al.* 2006) have been discovered. There is increasing evidence that locally produced BMPs play key roles in differentiation of the pituitary. For instance, BMP-4 promotes pituitary prolactinoma pathogenesis through crosstalk between the BMP-4-mediated Smads and the estrogen receptor (Paez-Pereda *et al.* 2003). BMP-4 also inhibits corticotrophic pathogenesis and Cushing's disease in adult pituitary tumor cells (Giacomini *et al.* 2006). Thus, the pituitary BMP system likely acts as a regulator not only for organogenesis and differentiation process of pituitary cells, but also for transformation and tumorigenesis of the differentiated pituitary cells. Here, we uncovered effects of the pituitary BMP system on GH reduction elicited by OCT and BRC treatments.

Materials and Methods

Reagents and supplies

A 1:1 mixture of Dulbecco's Modified Eagle Medium/Ham F-12 medium (DMEM/F12), penicillin-streptomycin solution, forskolin (FSK), and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma-Aldrich Corp. Recombinant human BMP-4 and BMP-6 were purchased from R&D Systems (Minneapolis, MN, USA) and activin A was from Sigma-Aldrich Corp. Control rat pituitary and ovary tissues were collected from 8-week-old female Wistar rats (CLEA Japan Inc., Tokyo, Japan). BRC mesylate and OCT acetate were provided by Novartis International Pharmaceutical Ltd (Basel, Switzerland). Plasmids of Id-1-Luc were gifts from Dr Tetsuro Watabe and Dr Kohei Miyazono, Tokyo University, Japan.

Cell culture and measurement of cAMP production

Rat pituitary somatotrophic tumor GH3 cells were provided from Prof. Joseph Majzoub, Children's Hospital, Harvard Medical School. Cells were cultured in DMEM/F12

medium supplemented with 10% fetal calf serum (FCS) and antibiotics in a 5% CO₂ atmosphere at 37 °C. GH3 cells (1×10^5 viable cells) were seeded in 24-well plates with DMEM/F12 containing 10% FCS and penicillin-streptomycin. After preculture, the medium was changed into serum-free DMEM/F12 containing penicillin-streptomycin and 0.1 mM IBMX (a specific inhibitor of phosphodiesterase activity), and then cells were treated with indicated concentrations and combinations of FSK, BRC, OCT, BMP-4, BMP-6, and activin A. After indicated culture periods, the supernatant of the culture media was collected and stored at -80 °C until assay. The extracellular contents of cAMP were determined by enzyme immunoassay (Assay Designs Inc., Ann Arbor, MI, USA) after the acetylation of each sample with assay sensitivity of 0.039 nM.

Determination of GH and prolactin levels

The GH3 cells (1×10^5 viable cells) were cultured in 24-well plates with DMEM/F12 containing 10% FCS and penicillin-streptomycin. After preculture, the medium was changed into serum-free DMEM/F12, and then cells were treated with indicated concentrations and combinations of FSK, BRC, OCT, BMP-4, BMP-6, and activin A. After 24-h culture, the supernatant of the culture media was collected and stored at -80 °C until assay. The levels of GH and prolactin in the cultured media were determined by rat-specific enzyme immunoassay (Duhau *et al.* 1991, Ezan *et al.* 1997; SPI-BIO, Montigny-le-Bretonneux, France).

RNA extraction, RT-PCR, and quantitative real-time PCR analysis

After preculture, cells (3×10^5 viable cells) were treated with indicated concentrations of FSK, BRC, and OCT in serum-free DMEM/F12. After 24-h culture, the medium was removed and total cellular RNA was extracted using TRIzol (Invitrogen Corp). The whole rat pituitary and ovary tissues were homogenized and total tissue RNA was extracted using TRIzol. Total RNA was quantified by measuring the absorbance of the sample at 260 nm, and stored at -80 °C until assay. The expression of dopamine D2 receptor (D2R), somatostatin receptors (SSTRs), BMP/activin ligands, BMP/activin receptors, Smads, a BMP-binding protein follistatin (FST), and housekeeping gene ribosomal L19 (RPL19) mRNAs were detected by reverse transcription-PCR (RT-PCR) analysis. The extracted RNA (1 μ g) was subjected to a RT reaction using First-Strand cDNA Synthesis System (Invitrogen Corp) with random hexamer (2 ng/ μ l), reverse transcriptase (200 U), and deoxyNTP (dNTP; 0.5 mM) at 42 °C for 50 min, 70 °C for 10 min. Subsequently, hot start PCR was performed using MgCl₂ (1.5 mM), dNTP (0.2 mM), and 2.5 U of Taq DNA polymerase (Invitrogen Corp). Oligonucleotides used for RT-PCR were custom ordered from Invitrogen Corp. PCR primer pairs were selected from different exons of the

corresponding genes as follows: D2R: 542–562 and 851–871 (from GenBank accession No. X56065); SSTR1: 526–546 and 869–889 (X62314); SSTR2: 240–260 and 559–579 (M93273); SSTR3: 501–521 and 782–802 (X63574); SSTR4: 400–420 and 781–801 (M96544); SSTR5: 98–118 and 368–388 (L04535); BMP-2: 198–218 and 468–488 (Z25868); BMP-4: 564–584 and 784–804 (NM_012827); BMP-6: 1042–1061 and 1246–1265 (AY184240); BMP-7: 418–438 and 684–704 (X56906); activin β A: 488–503 and 647–667 (NM_017128); activin β B: 427–446 and 634–653 (XM_001053684); inhibin α : 214–233 and 394–414 (BC083564); activin receptor-like kinase (ALK)-4: 1098–1118 and 1521–1541 (NM_007395); activin type II receptor B (ActRIIB): 301–325 and 548–571 (NM_031554); Smad5: 864–886 and 1062–1083 (NM_008541); Primer pairs for ALK-2, ALK-3, ALK-6, ActRII, BMP type II receptor (BMPRII), Smad1, Smad2, Smad3, Smad4, Smad6, Smad7, Smad8, FST, and RPL19 were selected as we reported earlier (Otani *et al.* 2007). Aliquots of PCR products were electrophoresed on 1.5% agarose gels and visualized after ethidium bromide staining. For the quantification of indicated mRNA levels, real-time PCR was performed using LightCycler-FastStart DNA Master SYBR Green I system (Roche Diagnostic Co.) under conditions of annealing at 60 °C with 4 mM MgCl₂, following the manufacturer's protocol. Accumulated levels of fluorescence were analyzed by the second-derivative method after the melting curve analysis (Roche Diagnostic), and then the expression levels of target genes were standardized by RPL19 level in each sample.

Immunofluorescence microscopy

GH3 cells were precultured in serum-free DMEM/F12 using chamber slides (Nalge Nunc Int., Naperville, IL, USA) and cells at ~50% confluency were treated with BMP-4 (100 ng/ml). After 1-h stimulation, the cells were fixed with 4% formaldehyde in PBS, permeabilized with 0.5% Triton X-100 in PBS at room temperature, and washed three times with PBS. The cells were then incubated with anti-phospho-Smad1,5,8 antibody (Cell signaling Technology Inc., Beverly, MA, USA) for 1 h and washed three times with PBS. The cells were then incubated with Alexa Fluor 488 anti-rabbit IgG (Invitrogen Corp) in humidified chamber for 1 h and washed with PBS, and then stained cells were visualized under a fluorescent microscope.

Western immunoblot analysis

The cells (3×10^5 viable cells) were cultured in 24-well plates in DMEM/F12 containing penicillin–streptomycin. After preculture, the medium was changed into serum-free DMEM/F12 and cultured for 24 h, and then the cells were treated with indicated concentrations and combinations of OCT, BRC, and BMP-4. After 1- and 3-h culture, the cells were solubilized in 100 μ l RIPA lysis buffer (Upstate

Biotechnology Inc., Lake Placid, NY, USA) containing 1 mM Na₃VO₄, 1 mM NaF, 2% SDS, and 4% β -mercaptoethanol. The cell lysates were then subjected to SDS-PAGE immunoblotting analysis using anti-phospho-Smad1,5,8 antibody (Cell signaling Technology Inc.).

Transient transfection and luciferase assay

The cells (1×10^5 viable cells) were cultured in 24-well plates in DMEM/F12 with 10% FCS containing penicillin–streptomycin. The cells were then transiently transfected with 250 ng each luciferase reporter plasmid (Id-1-Luc) and 25 ng cytomegalovirus- β -galactosidase plasmid (pCMV- β -gal) using FuGENE6 (Roche Molecular Biochemicals). After 16-h incubation, the culture medium was changed into serum-free DMEM/F12 and then the cells were treated with indicated concentrations of BRC and OCT in the presence or absence of BMP-4 for 24 h. The cells were washed with PBS and lysed with Cell Culture Lysis Reagent (TOYOBO, Osaka, Japan). Luciferase activity and β -galactosidase (β -gal) activity of the cell lysate were measured by luminometer. The data were shown as the ratio of luciferase and β -gal activities.

Statistical analysis

All results are shown as mean $\pm$ S.E.M. of data from at least three separate experiments, each performed with triplicate samples. Differences between groups were analyzed for statistical significance using ANOVA with Fisher's protected least significant difference (PLSD) test (StatView 5.0 software, Abacus Concepts Inc., Berkeley, CA, USA). Values of $P < 0.05$ were accepted as statistically significant.

Results

We first examined expression profile of the BMP system in rat pituitary tumor GH3 cells and rat normal pituitary tissues by RT-PCR (Fig. 1). Rat ovarian tissue RNA was utilized as a positive control. The GH3 cells expressed SSTRs including all of SSTR1, 2, 3, 4, and 5 and dopamine receptor D2R as reported previously (Johnston *et al.* 1991, Yang *et al.* 2005). The expression level of SSTR2 in GH3 cells was relatively high when compared with that in rat pituitary tissues, while D2R transcript was comparably low in GH3 cells. In addition, both GH3 cells and rat whole pituitaries expressed BMP/activin type-1 (ALK-2, ALK-3, and ALK-4), type-2 (ActRII, ActRIIB, and BMPRII) receptors and Smads (Smad1, Smad2, Smad3, Smad4, Smad5, Smad6, Smad7, and Smad8). Neither GH3 cells nor rat whole pituitaries expressed the BMP type-1B receptor, ALK-6. FST expression was not detected in GH3 cells although it was expressed in the whole pituitary. The whole pituitary expressed all BMP/activin ligands examined, including BMP-2, BMP-4, BMP-6, BMP-7, activin β A/ β B, and inhibin α subunits.

Figure 1 Expression of BMP system and somatostatin receptors in GH3 cells. Total cellular RNA was extracted from GH3 cells, rat whole pituitary, and rat whole ovary tissues. Total cellular RNA was quantified by measuring the absorbance of the sample at 260 nm. The expression of BMP/activin (BMP-2, BMP-4, BMP-6, BMP-7/activinβA, activinβB, and inhibinα) ligands, type-1 (ALK-2, ALK-3, ALK-4, and ALK-6) and type-2 (ActRII, ActRIIB, and BMPRII) receptors, a binding protein follistatin (FST), Smad1–8, somatostatin receptor (SSTR) 1–5, dopamine D2 receptor (D2R), and housekeeping gene RPL19 mRNAs was detected by RT-PCR analysis. MM, molecular weight marker.

The predominant BMP ligands expressed in GH3 cells were BMP-4 and BMP-6 (Fig. 1).

To evaluate cellular responses with respect to GH secretion, we stimulated GH3 cells with FSK and determined the accumulated levels of cAMP and GH in the culture medium using enzyme immunoassays (Fig. 2). As shown in Fig. 2A, FSK (1–10 μM) induced cAMP production in GH3 cells for 24 h in a time- and concentration-dependent manner. In accordance with cAMP synthesis, FSK (1–10 μM) stimulated GH production concentration dependently for 24-h culture (Fig. 2B). We next examined the effect of OCT (0.03–10 μM) and BRC (0.3–100 μM) on FSK-induced cAMP production (Fig. 3). OCT showed a moderate but insignificant reduction of cAMP levels induced by FSK (1 μM), whereas BRC exhibited potent suppression of FSK-induced cAMP production by GH3 cells (Fig. 3A). When cells were treated with BRC in combination with OCT (Fig. 3B), lower concentrations of OCT (0.1–1 μM) exhibited additive effects on BRC-induced cAMP reduction. Interestingly, the effects of BRC were impaired when added in combination with a high concentration (10 μM) of OCT (Fig. 3B).

As shown in Fig. 4A, OCT (0.03–10 μM) and BRC (0.3–100 μM) independently reduced FSK-induced GH production. A significant reduction of prolactin secretion was also observed in BRC-treated GH3 cells (data not shown). When cells were treated with the combination of BRC and OCT, a low concentration (0.1 μM) of OCT exerted additive effects on the BRC effects suppressing FSK (1 μM)-induced GH levels (Fig. 4B). In contrast, a high concentration

(10 μM) of OCT impaired the BRC effects reducing FSK-induced GH production (Fig. 4B). The changes of GH reduction were in parallel with the changes of cAMP reduction induced by BRC and OCT (see Figs 3B and 4B).

To elucidate functional roles of the BMP system expressed in GH3 cells, we treated cells with BMP-4, BMP-6, and activin A in the presence or absence of FSK and determined GH and cAMP production (Fig. 5). As shown in Fig. 5A, BMP-4, BMP-6, and activin A (10–100 ng/ml) had no significant effects on basal GH production by GH3 cells. It was of note that BMP-4 increased GH production induced by FSK (1 μM) for 24-h culture (Fig. 5B). BMP-4 also increased FSK-induced cAMP synthesis by GH3 cells (Fig. 5C). In contrast, BMP-6 and activin A had no significant effects on GH and cAMP levels induced by FSK in GH3 cells (Fig. 5B and C).

The effects of OCT and BRC on BMP–Smad signaling were further examined by immunoblot and reporter-gene assays (Fig. 6). As shown in Fig. 6A, Smad1,5,8 phosphorylation was readily activated by BMP-4 (100 ng/ml) in GH3 cells. Immunofluorescence studies clearly demonstrated nuclear localization of the phosphorylated Smad1,5,8 molecules in response to BMP-4 addition. As shown by immunoblot analysis (Fig. 6A), BMP-4-induced Smad1,5,8 phosphorylation was not affected by adding OCT (10 μM); however, BRC (100 μM) inhibited BMP-4-induced Smad1,5,8 phosphorylation. Furthermore, transcription of the BMP-target gene, *Id-1*, was readily activated by BMP-4 (100 ng/ml; Fig. 6B). The *Id-1*-Luc activity induced by

Figure 2 Forskolin induction of cAMP and GH in GH3 cells. GH3 cells (1×10^5 viable cells) were precultured in serum-free DMEM/F12 and then treated with forskolin (FSK, 1–10 μ M). (A) The extracellular contents of cAMP in the medium were determined after 3–24-h of culture. (B) cAMP levels and GH concentrations in the medium were determined by specific enzyme immunoassays after 24-h culture. For measurement of cAMP levels, the cells were cultured with serum-free medium containing 0.1 mM IBMX. Results are shown as mean $\pm$ S.E.M. of data from at least three separate experiments, each performed with triplicate samples. * $P < 0.05$ and ** $P < 0.01$ versus control in each group or between the indicated groups.

Figure 3 Effects of octreotide and bromocriptine on forskolin-induced cAMP levels. GH3 cells (1×10^5 viable cells) were precultured in serum-free DMEM/F12. (A) Cells were treated with either octreotide (OCT, 0.03–10 μ M) or bromocriptine (BRC, 0.3–100 μ M) in the presence of forskolin (FSK, 1 μ M). (B) The cells were treated with OCT (0.1–10 μ M) in combination with BRC (1–100 μ M) in the presence of FSK (1 μ M). After 24-h treatment, extracellular cAMP levels in the culture medium containing 0.1 mM IBMX were determined by enzyme immunoassay. Results are shown as mean $\pm$ S.E.M. of data from at least three separate experiments, each performed with triplicate samples. * $P < 0.05$ versus control in each group or between the indicated groups.

BMP-4 was not affected by OCT (0.1–100 μ M), whereas it was significantly suppressed by BRC treatment (1–100 μ M) in a concentration-dependent manner (Fig. 6B). As shown in Fig. 6C, a low concentration of OCT (0.1 μ M) had additive effects on the BRC suppression of Id-1-Luc activity induced by BMP-4. However, the BRC suppression of BMP-4-induced Id-1-Luc activity was impaired in the presence of a high concentration (10 μ M) of OCT (Fig. 6C).

We further examined the combination effects of OCT and BRC on cAMP and GH production in the presence of BMP-4 (Fig. 7). As shown in Fig. 7A, BMP-4 (100 ng/ml) enhanced FSK (1 μ M)-induced cAMP levels, which was dose dependently suppressed by BRC treatment. In GH3 cells activated by FSK and BMP-4, a low concentration of OCT (0.1 μ M) had additive effects on the cAMP reduction evoked by BRC, whereas a high concentration (10 μ M) of OCT rather impaired the BRC effects reducing cAMP synthesis (Fig. 7A). Likewise,

BMP-4 (100 ng/ml) enhanced FSK (1 μ M)-induced GH levels and BRC treatment reduced the GH production stimulated by FSK and BMP-4 (Fig. 7B). Furthermore, a low concentration of OCT (0.1 μ M) facilitated BRC-induced GH reduction in GH3 cells activated by FSK and BMP-4, whereas a high concentration (10 μ M) attenuated the BRC effects reducing GH production (Fig. 7B).

To assess the possible mechanism by which OCT and BRC affect BMP-4 signaling in GH3 cells, changes of mRNA levels of endogenous BMP ligands including BMP-4, BMP-6; ALK-2, ALK-3; ActRII; BMPRII, and inhibitory Smad6 and Smad7 were examined by quantitative PCR (Fig. 8). FSK (1 μ M) treatment had no significant effects on the expression of BMP ligands or BMP signaling molecules in GH3 cells. In the presence of FSK, BRC (100 μ M) caused a decrease in ALK-3 expression levels, while OCT had no significant effects on the expression levels of these BMP molecules examined.

Figure 4 Effects of octreotide and bromocriptine on forskolin-induced GH production by GH3 cells. GH3 cells (1×10^5 viable cells) were precultured in serum-free DMEM/F12. (A) The cells were treated with either octreotide (OCT, 0.03–10 μM) or bromocriptine (BRC, 0.3–100 μM) in the presence of forskolin (FSK, 1 μM). (B) The cells were treated with OCT (0.1–10 μM) in combination with BRC (1–100 μM) in the presence of FSK (1 μM). And then, GH levels in the culture medium for 24-h treatment were determined by enzyme immunoassay. Results are shown as mean $\pm$ S.E.M. of data from at least three separate experiments, each performed with triplicate samples. * $P < 0.05$ and ** $P < 0.01$ versus control in each group or between the indicated groups.

Discussion

In the present study, we found that BMP-4 facilitates FSK-induced cAMP and GH production through BMP receptor and Smad1,5,8 pathway of GH3 cells (Fig. 9). BRC suppressed FSK-induced cAMP synthesis, leading to the reduction of GH secretion, whereas OCT reduced GH production possibly by a cAMP-independent mechanism.

The efficacy of somatostatin analogs is linked to the SSTR selectivity profile, in which binding capability to SSTR2 and SSTR5 appears critical (Shimon *et al.* 1997). SSTR2 and SSTR5 are negatively coupled to adenylyl cyclase, activation of which results in a reduction of intracellular cAMP concentrations (Reisine & Bell 1995). The SSTR effects are further mediated by Ca^{++} influx through a direct action on Ca^{++} channels (Chen *et al.* 1997) and/or indirectly through

Figure 5 Effects of BMP-4 on GH and cAMP production. GH3 cells (1×10^5 viable cells) were precultured in serum-free DMEM/F12. The cells were then treated with BMP-4 (10–100 ng/ml), BMP-6 (10–100 ng/ml), and activin A (10–100 ng/ml) in the presence or absence of forskolin (FSK, 1 μM). After 24-h culture, the culture media were collected and (A and B) GH production and (C) cAMP levels were determined by specific enzyme immunoassays. For measurement of cAMP levels, the cells were cultured with serum-free medium containing 0.1 mM IBMX. Results are shown as mean $\pm$ S.E.M. of data from at least three separate experiments, each performed with triplicate samples. * $P < 0.05$ and ** $P < 0.01$ versus control in each group.

Figure 6 Effects of octreotide and bromocriptine on BMP-4-Smad signaling. (A) GH3 cells were precultured in serum-free DMEM/F12 using chamber slides and treated with BMP-4 (100 ng/ml) for 1 h. The cells were then fixed, permeabilized, and incubated with anti-phospho-Smad1,5,8 (pSmad1,5,8) antibody for immunofluorescent (IF) studies. For immunoblotting analysis, GH3 cells (3×10^5 viable cells) were precultured in serum-free DMEM/F12. After pretreatment with octreotide (OCT, 10 μM) or bromocriptine (BRC, 100 μM), the cells were treated with BMP-4 (100 ng/ml) for 60–180 min. The cell lysates were then subjected to SDS-PAGE immunoblotting (IB) analysis using anti-pSmad1,5,8 antibody. (B) GH3 cells (1×10^5 viable cells/ml) were transiently transfected with Id-1-Luc and pCMV-β-gal. The cells were then treated with either OCT (0.1–100 μM) or BRC (1–100 μM) in the presence of BMP-4 (100 ng/ml) for 24 h. (C) GH3 cells transiently transfected with Id-1-Luc and pCMV-β-gal were treated with OCT (0.1–10 μM) in combination with BRC (1–100 μM) in the presence of BMP-4 (100 ng/ml) for 24 h. Luciferase activity and β-galactosidase (β-gal) activity of the cell lysates were measured. The data were shown as the ratio of luciferase to β-gal activity. Results are shown as mean $\pm$ S.E.M. of data from at least three separate experiments, each performed with triplicate samples. * $P < 0.05$ and ** $P < 0.01$ versus control in each group.

activating K^+ channels (Takano *et al.* 1997; Fig. 9). On the other hand, D1 and D2 dopamine receptors are widely distributed in the central nervous system and the gastrointestinal tract. D2 dopamine receptors are the predominant mediators of GH regulation in the pituitary. Since

Figure 7 Effects of octreotide and bromocriptine on BMP-4 enhancement of cAMP and GH production induced by forskolin. GH3 cells (1×10^5 viable cells) were precultured in serum-free DMEM/F12. The cells were treated with octreotide (OCT, 0.1–10 μM) in combination with bromocriptine (BRC, 1–100 μM) under the presence of BMP-4 (100 ng/ml) and forskolin (FSK, 1 μM). After 24-h treatment, (A) extracellular cAMP levels and (B) GH production in culture medium were determined by enzyme immunoassays. For measurement of cAMP levels, the cells were cultured with serum-free medium containing 0.1 mM IBMX. Results are shown as mean $\pm$ S.E.M. of data from at least three separate experiments, each performed with triplicate samples. * $P < 0.05$ and ** $P < 0.01$ versus control in each group or between the indicated groups.

GH-producing adenomas having a high activity of adenyl cyclase exhibit more sensitivity to dopamine agonists (Spada *et al.* 1990), the capability of cAMP suppression is likely to be a key factor for GH reduction by dopamine agonists.

Combination treatment with somatostatin analogs and dopamine agonists is reserved for acromegaly patients who are resistant to single medical treatment. However, the mechanism of action underlying the efficaciousness of the combined treatment has been uncertain. Balsa and colleagues reported interesting findings regarding the effects of combination therapy on GH release in cultures of surgically removed GH-producing adenomas (Balsa *et al.* 2002). In their

Figure 8 Effects of octreotide and bromocriptine on BMP system expression in GH3 cells. GH3 cells (3×10^5 viable cells) were cultured in serum-free DMEM/F12 with forskolin (FSK, 1 μ M), octreotide (OCT, 0.1–10 μ M), and bromocriptine (BRC, 1–100 μ M). After 24-h culture, total cellular RNA was extracted and subjected to RT-PCR. The mRNA expression levels of BMP-4, BMP-6, ALK-2, ALK-3, ActRII, BMPRII, Smad6, and Smad7 were quantified by real-time PCR analysis. The expression levels of target genes were standardized by RPL19 levels in each sample. Results are shown as mean $\pm$ S.E.M. of data from at least three separate experiments, each performed with triplicate samples. * $P < 0.05$ versus control in each group.

study, the combination treatment with somatostatin analogs and BRC was found to be effective in 18–30% tumor cell cultures. However, the significant correlation between cAMP levels and GH reduction in the cultures were not clearly displayed, implying the involvement of other pathways than adenylyl cyclase–cAMP signaling in the mechanism of GH reduction by the combination treatment.

In the present study, a novel role of endogenous BMP-4 mediating the effects of OCT and BRC combination treatment with respect to GH production was elucidated in the GH3 cell model (Fig. 9). Specifically, BMP-4 stimulated GH and cAMP synthesis induced by FSK. Since

GH-releasing hormone elicits GH synthesis and secretion by activating cAMP in the pituitary somatotrope, this inter-relationship between cAMP and BMP-4 would be physiologically critical in regulating GH secretion by the normal pituitary and GH-producing adenomas. Importantly, BRC but not OCT inhibited BMP-4-induced activation of Smad1,5,8 phosphorylation and Id-1 transcription. The reduction of ALK-3 expression may be involved in this mechanism. Furthermore, in cases of combination treatment with OCT and BRC, addition of a high concentration of OCT resulted in decreasing the major BRC effects including the inhibition of BMP-4 signaling and the reduction of

Figure 9 A possible involvement of BMP-4 in regulating GH secretion by octreotide and bromocriptine. BMP-4 facilitates forskolin (FSK)-induced cAMP and GH production through BMP receptors, ALK-3/BMPRII, and Smad1,5,8 pathway of GH3 cells. Bromocriptine (BRC) suppresses FSK-induced cAMP synthesis, leading to the reduction of GH secretion. Octreotide (OCT) reduces GH production possibly by cAMP-independent mechanism. Importantly, exposure to a high concentration of OCT impairs the BRC effects suppressing FSK-induced cAMP production and BMP-4-Smad1,5,8 signaling in GH3 cells. AC, adenylyl cyclase; PKA, protein kinase-A.

FSK-induced cAMP production. These findings suggested the involvement of endogenous BMP-4 actions in controlling FSK-induced GH levels by OCT and BRC.

BMP-2 and BMP-4 have been known to play a key role in the initial development of the anterior pituitary (Scully & Rosenfeld 2002). BMP-4 is required during the first stage of pituitary organogenesis for the proliferation of the Rathke's pouch that gives rise to Pit-1 lineage cells including lactotrope cells. Overexpression of noggin or a dominant-negative ALK-3 in the anterior pituitary leads to the arrest of the development of Pit-1-expressing lineage (Scully & Rosenfeld 2002). During the following stages of pituitary organogenesis, an inhibition of BMP-2 by fibroblast growth factor-8 leads to differentiation of corticotrope cells (Kioussi *et al.* 1999, Dasen & Rosenfeld 2001). BMP-4 not only governs this pituitary organogenesis but also plays a key role in the pathogenesis of differentiated pituitary lineages. For instance, BMP-4 is overexpressed in lactotrope adenomas derived from D2R-null mice as well as human prolactinomas (Paez-Pereda *et al.* 2003). A binding protein for BMPs, noggin expression is conversely downregulated in the prolactinoma from the D2R-null mouse (Paez-Pereda *et al.* 2003), suggesting that BMP-4 promotes cell proliferation in lactotropes in conjunction with Smad-estrogen receptor interaction under the influence of its binding protein. BMP-4 also inhibits

adrenocorticotropin secretion and cell proliferation of corticotropinoma cells (Giacomini *et al.* 2006). This BMP-4 action seems to be involved in the anti-proliferative effect induced by retinoic acid on corticotropinomas. Thus, BMP-4 promotes pituitary prolactinoma through Smad-estrogen receptor crosstalk (Paez-Pereda *et al.* 2003) while BMP-4 inhibits corticotrope pathogenesis of Cushing's disease (Giacomini *et al.* 2006).

Several preferential combinations of BMP ligands and receptors have been recognized to date, for example, BMP-2 and BMP-4 preferentially bind to ALK-3 and/or ALK-6, BMP-6, and BMP-7 most readily bind to ALK-2 and/or ALK-6 (ten Dijke *et al.* 1994, Yamashita *et al.* 1995, Ebisawa *et al.* 1999, Aoki *et al.* 2001), and BMP-15 efficiently binds to ALK-6 with much lower affinity for ALK-3 (Moore *et al.* 2003). Regarding type II receptors, ActRII, which was originally identified as activin receptors, also acts as receptors for BMP-6 and BMP-7 (Yamashita *et al.* 1995, Ebisawa *et al.* 1999), while BMPRII binds exclusively to BMP ligands including BMP-2, BMP-4, BMP-6, BMP-7, and BMP-15 (Liu *et al.* 1995, Nohno *et al.* 1995, Rosenzweig *et al.* 1995, Moore *et al.* 2003). Since ALK-6 is not expressed in GH3 cells, the receptor pairs of ALK-3 and BMPRII are likely to be the major functional complex for BMP-4 for activating cAMP and GH production by GH3 cells. The reduction of ALK-3 expression may be involved, at least in part, in the mechanism by which BRC suppresses BMP-4 signaling in GH3 cells. Notably, the exposure to a high concentration of OCT impairs the BRC effects suppressing cAMP-to-GH production induced by FSK and BMP-4 as well as BMP-4-Smad1,5,8 signaling in GH3 cells. However, the molecular mechanism by which a high concentration of OCT inactivates BRC actions has yet to be elucidated. In this regard, recent studies have shown the presence of a heterodimeric association between SSTR5 and D2R, leading to the enhanced biological activity (Rocheville *et al.* 2000). Further investigation is necessary to approach the impact and possibility of the receptor heteromerization of OCT and BRC on GH regulation and its interaction with pituitary BMP system.

Collectively, a new interaction between cAMP and BMP-4 signaling for GH production was uncovered. Treatment with high concentrations of OCT interferes with key BRC effects that inhibit cAMP production as well as the BMP-4 pathway *in vitro*. This interaction may be involved in the mechanism of ineffectiveness of GH reduction in combination therapy with OCT and BRC for acromegaly patients when treated with high concentrations of OCT.

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