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Novel Action of Activin and Bone Morphogenetic Protein in Regulating Aldosterone Production by Human Adrenocortical Cells

JIRO SUZUKI, FUMIO OTSUKA, KENICHI INAGAKI, MASAYA TAKEDA, TOSHIO OGURA, AND HIROFUMI MAKINO

Department of Medicine and Clinical Science, Okayama University Graduate School of Medicine and Dentistry, Okayama City 700-8558, Japan

We have uncovered a functional bone morphogenetic protein (BMP) and activin system complete with ligands (BMP-6 and activin βA/βB), receptors (activin receptor-like kinase receptors 2, 3, and 4; activin type-II receptor; and BMP type-II receptor), and the binding protein follistatin in the human adrenocortical cell line H295R. Administration of activin and BMP-6 to cultures of H295R cells caused concentration-responsive increases in aldosterone production. The mRNA levels of steroidogenic acute regulatory protein or P450 steroid side-chain cleavage enzyme, the rate-limiting steps of adrenocortical steroidogenesis, were enhanced by activin and BMP-6. Activin and BMP-6 also activated the transcription of steroidogenic acute regulatory protein as well as the late-step steriodogenic enzyme CYP11B2. Activin enhanced ACTH-, forskolin-, or dibutyryl-cAMP- but not angiotensin II (Ang II)-induced aldosterone production, whereas BMP-6 specifically augmented Ang II-induced aldosterone production. Activin and ACTH but not BMP-6 increased cAMP production. Follistatin, which inhibits activin actions by binding, suppressed basal and ACTH-induced aldosterone secretion but failed to affect the Ang II-induced aldosterone level. Furthermore, MAPK signaling appeared to be involved in aldosterone production induced by Ang II and BMP-6 because an inhibitor of MAPK activation, U0126, reduced the level of aldosterone synthesis stimulated by Ang II and BMP-6 but not activin. In addition, Ang II reduced the expression levels of BMP-6 but increased that of activin βB, whereas ACTH had no effect on these levels. Collectively, the present data suggest that activin acts to regulate adrenal aldosterone synthesis predominantly by modulating the ACTH-cAMP-protein kinase A signaling cascade, whereas BMP-6 works primarily by modulating the Ang II-MAPK cascade in human adrenal cortex in an autocrine/paracrine fashion. (*Endocrinology* 145: 639–649, 2004)

THE SYSTEMIC HOMEOSTASIS of electrolytes and fluid volume is strictly controlled by an endocrine system composed of renin, angiotensin II (Ang II), and aldosterone (Aldo), *i.e.* the R-A-A system (1). In particular, Aldo is a key regulator of circulating blood volume and blood pressure and has also recently been implicated in promoting tissue remodeling in the cardiovascular system (2, 3). Production of Aldo occurs primarily in the adrenal glomerulosa and is rigorously controlled by Ang II, ACTH, and K^+. The precise regulation of Aldo synthesis is dependent upon their combined activities (1, 4).

Synthesis of Aldo can be divided into two distinct phases, the acute phase and the chronic phase (1, 4). The acute phase occurs within minutes and involves the transfer of cholesterol to the mitochondria where it is cleaved by P450 side-chain cleavage enzyme (P450scc) (5), followed by the conversion of pregnenolone to progesterone. The chronic phase is marked by an increase of Aldo synthase (P450aldo) expression. P450aldo catalyzes the multistep process by which 11-deoxycorticosterone (DOC) is converted to corticosterone and finally to Aldo. In the acute stage, Ang II, ACTH, and K^+ can all enhance the early steps of steroid biosynthesis, whereas during long-term experiments, the later stages of Aldo synthesis are promoted primarily through Ang II. The major signal transduction pathway for ACTH stimulation of Aldo production occurs through cAMP/protein kinase A (PKA), and Ang II action is transduced through the diacylglycerol/protein kinase C and inositol 1,4,5-trisphosphate/Ca^{2+} signaling mechanism (6) via the Ang II type-I (AT1) receptor (4, 7). Additionally, MAPK-signaling molecules have also been implicated in Ang II stimulation of Aldo synthesis (8–11).

There is accumulating evidence that several growth factors and cytokines can act as local autocrine/paracrine regulators for modulating the activity of the hormones that stimulate steroidogenesis by the adrenal cortex (7). Among these factors, basic fibroblast growth factor, IGFs, and TGF-β1 have been postulated to play roles in regulating adrenal steroidogenesis (7, 12–14). For instance, TGF-β1 has been shown to act as a potent inhibitor of basal and hormone-induced steroidogenesis in the adrenal cortex (15).

Activins and inhibins, which also belong to the TGF-β superfamily (16), are dimeric glycoproteins formed by two of

Abbreviations: ActR-II, Activin type-II receptor; Aldo, aldosterone; ALK, activin receptor-like kinase; Ang II, angiotensin II; AT1, angiotensin type-1; BMP, bone morphogenetic protein; BMPR-II, BMP type-II receptor; BtcAMP, N^6,$O^{2'}$-dibutyryl-cAMP; DOC, deoxycorticosterone; FSK, forskolin; IBMX, 3-isobutyl-1-methylxanthine; 12-LO, 12-lipoxygenase; P450aldo, P450 aldosterone synthase; P450scc, P450 steroid side-chain cleavage enzyme; pCMV-β-gal, cytomegalovirus-β-galactosidase plasmid; PDGF, platelet-derived growth factor; PKA, protein kinase A; StAR, steroidogenic acute regulatory protein.

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three different subunits (α, βA, and βB). Activins are produced in numerous cell types and organs and act as autocrine/paracrine regulators of a wide variety of cellular functions. In contrast, inhibin production is quite restricted to steroidogenic tissues (17) and often acts hormonally in addition to its local actions. Activin/inhibin receptors and the binding protein follistatin are expressed in the human adrenal gland and have been shown to regulate inhibin secretion through an autocrine/paracrine mechanism (18).

Bone morphogenetic proteins (BMPs) were identified in 1965 by Urist as the active components in demineralized bone and bone extracts that are capable of inducing bone formation at ectopic sites (19). Shortly after the cloning of the first BMPs, a number of new BMP family genes were identified by using homology-based cDNA cloning. BMPs regulate cell growth, apoptosis, differentiation, and cell patterning and specification in numerous tissues (20). Recent studies have shown that BMPs also exhibit multifunctional activities in endocrine tissues including the ovary (21–23) and the pituitary (24, 25). Currently, there are no data as to whether the BMP system plays a role in adrenal function.

The receptors for TGF-β superfamily members consist of type-I and type-II receptors with serine/threonine kinase (26, 27). Activin and TGF-β ligands first bind to the type-II receptors followed by the recruitment of type-I receptors. As for BMPs, BMPR-II and the appropriate type-I receptor act together to form a high-affinity complex. The combinations of BMP ligands and receptors are not exclusive; *e.g.* activin type-II receptors (ActR-II and -IIB) can act for BMP-6 binding whereas BMPR-II can bind specifically to BMP ligands. Regarding type-I receptors, activin receptor-like kinase (ALK)-2 (also called ActR-IA), ALK-3 (BMPR-IA), and ALK-6 (BMPR-IB) have been identified as type-I receptors for BMPs whereas ALK-4 (ActR-IB) is the type-I receptor for activin (26, 28). Some preferential combinations seem to exist between BMP ligands and type-I receptors despite the cross-reactivity among ligands and type-I receptors, in which BMP-6 most readily binds to ALK-2 and/or ALK-6, although there are some cell-dependent differences on the binding affinities to the specific type-I receptors (29, 30).

Here we demonstrate the existence of functional activin and BMP systems in human adrenocortical H295R cells, which originated from a human adrenocortical carcinoma and are established as an appropriate model to define mechanisms regulating Aldo production (31, 32). The present study demonstrates novel actions of activin/BMPs in regulating the synthesis of Aldo. The mechanism involving the activin/BMP system may play critical roles in the development and/or progression of functional adrenal tumors that cause primary aldosteronism or Cushing's syndrome.

Materials and Methods

Reagents

A 1:1 mixture of DMEM/Ham's F-12 medium (DMEM/F12), penicillin-streptomycin solution, Ang II acetate salt, ACTH human fragment 1–24 (1–24 ACTH), forskolin (FSK), and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Recombinant human platelet-derived growth factor (PDGF)-BB and human TGF-β1 were from PeproTech EC Ltd. (London, UK), NuSerum and insulin-transferrin-sodium selenite Plus (ITS+) from BD Falcon (Bedford, MA), and recombinant human BMP-6 from R&D Systems (Minneapolis, MN). Recombinant human activin-A and follistatin were a kind gift from Dr. Shunichi Shimasaki. N^6,$O^{2'}$-Dibutyryl-cAMP monosodium salt (BtcAMP) was from Yamasa-shoyu (Tokyo, Japan). Human ovary total RNA was purchased from Stratagene Inc. (La Jolla, CA), and U0126 was purchased from Promega Corp. (Madison, WI). Plasmids of pGL2-basic and pGL2-steroidogenic acute regulatory protein (pGL2-StAR) (33) were kindly provided from Dr. Jerome F. Strauss III; pGL3-basic, pGL3-CYP11B1 (34), and pGL3-CYP11B2 (35) from Dr. William E. Rainey; Tlx2-Luc (36) from Dr. Jeff Wrana; and 3TP-Luc (37) and $(CAGA)^9$-Luc (38) from Dr. Kohei Miyazono.

Cell culture

The NCI-H295R human adrenocortical cell line was obtained from American Type Culture Collection (Manassas, VA). H295R cells were cultured in DMEM/F12 medium containing 2.5% NuSerum, 1% ITS+ supplements, and antibiotics (penicillin and streptomycin). The cells were cultured at 37 C under a humid atmosphere of 95% air/5% CO_2 as previously reported (31).

Aldo assay

To assess the effect of treatments on Aldo secretion, monolayered cells (~80% confluency) were precultured in 24-well human fibronectin-coated plates (Biocoat, BD-Falcon), and after 48 h of culture, the medium was replaced with fresh medium containing 0.3% NuSerum either alone or with a combination of the reagents including Ang II, ACTH, BMP-6, activin, BtcAMP, FSK, and U0126 at indicated concentrations. H295R cells were then cultured for another 48 h, and the accumulated levels of Aldo in the conditioned media were determined by RIA using the SPAC-S aldosterone kit (TFB Co., Tokyo, Japan).

Human adrenocortical tumors

Human adrenal RNA samples were collected from adrenocortical tumor tissues from patients who had been diagnosed, using clinical and pathological criteria, with aldosterone adenoma (primary aldosteronism; 50-yr-old male) or cortisol adenoma (Cushing's syndrome; 45-yr-old female). The tissues were immediately frozen in liquid nitrogen and stored at −80 C until extraction of the RNA. Total tissue RNAs were extracted by isothiocyanate-acid phenol-chloroform methods using TRIzol (Invitrogen Corp., Carlsbad, CA) after the tissue homogenization. All human subject protocols were approved by our institutional committee, and written permission from each individual regarding the experimental use of the tissues was obtained in advance of the surgery.

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

H295R cells were grown in six-well plates to approximately 80% confluence, then the medium was replaced with low-serum medium containing 0.3% NuSerum. The cells were treated with Ang II (10 nM), ACTH (100 ng/ml), BMP-6 (100 ng/ml), activin (100 ng/ml), BtcAMP (1 mM), or FSK (10 μM). After 48 h of culture, the medium was removed and total cellular RNA was extracted by isothiocyanate-acid phenol-chloroform methods using TRIzol (Invitrogen) and quantified by measuring absorbance at 260 nm and stored at −80 C until assay. The expression of BMP/activin ligands, receptors, follistatin, StAR, and P450scc mRNAs were detected by RT-PCR analysis. Oligonucleotides used for RT-PCR were custom-ordered from Kurabo Biomedical Co. (Osaka, Japan). PCR primer pairs were selected from different exons of the corresponding genes to discriminate PCR products that might arise from possible chromosome DNA contaminants. The extracted RNA (1 μg) was subjected to a RT reaction using the First-Strand cDNA synthesis system (Invitrogen) with random hexamer (2 ng/μl), reverse transcriptase (200 U), and deoxynucleotide triphosphate (0.5 mM) at 42 C for 50 min and 70 C for 10 min. Subsequently, hot-start PCR was performed using $MgCl_2$ (1.5 mM), deoxynucleotide triphosphate (0.2 mM), and 2.5 U of *Taq* DNA polymerase (Invitrogen) under the conditions we have previously reported (39). All the PCR product sizes are as follows: ALK-2, 706 bp; ALK-3, 510 bp; ALK-4, 529 bp; ALK-6, 456 bp; BMPR-II, 522 bp; ActR-II, 492 bp; follistatin, 188 bp; BMP-4, 400 bp;

BMP-6, 402 bp; BMP-7, 386 bp; BMP-15, 413 bp; activin βA, 388 bp; activin βB, 243 bp; and L19, 195 bp. Aliquots of PCR products were electrophoresed on 1.5% agarose gels and visualized after ethidium bromide staining. Total human ovary and adrenal RNAs (Stratagene, San Diego, CA) were used as control studies. For the quantification of StAR, P450scc, activin βB, BMP-6, and L19 mRNA levels, real-time PCR was performed using the LightCycler-FastStart DNA master SYBR Green I system (Roche Diagnostic Co., Tokyo, Japan) under the condition of annealing at 60 C with 4 mM $MgCl_2$ following the manufacturer's protocol. Accumulated levels of fluorescence were analyzed by fit-point method after the melting-curve analysis, and then the expression levels of StAR, P450scc, activin βB, and BMP-6 were standardized by the L19 level in each sample.

Transient transfection and luciferase assay

After a 24-h preculture in 12-well human fibronectin-coated plates (Biocoat, BD-Falcon), H295R cells (~70% confluency) were transiently transfected with 1 μg of each luciferase reporter plasmid [pGL2-StAR (–1.3 kb), pGL3-CYP11B1 (–1.5 kb), pGL3-CYP11B2 (–1.1 kb), Tlx2-Luc, 3TP-Luc, and $(CAGA)^9$-Luc] and 0.1 μg of cytomegalovirus-β-galactosidase plasmid (pCMV-β-gal) using FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN) for 24 h. The cells were then treated with indicated concentrations of activin and BMP-6 in DMEM/F12 containing 0.3% NuSerum for 24 h. The cells were washed with PBS and lysed with cell culture lysis reagent (TOYOBO, Osaka, Japan). Luciferase activity and β-gal activity of the cell lysate were measured by a TD-4000 luminometer (Turner Designs, Sunnyvale, CA). The data were shown as the ratio of luciferase to β-gal activity.

Measurement of cAMP production

To assess the effect of treatments on cAMP synthesis, monolayered H295R cells (1×10^5 viable cells) were precultured in 96-well plates, and 24 h later, the medium was replaced with fresh medium containing 0.3% NuSerum with activin, BMP-6, ACTH, or FSK at indicated concentrations in the presence of 0.1 mM IBMX (specific inhibitor of phosphodiesterase activity). After 48 h, the conditioned medium was collected and the extracellular content of cAMP was determined by a cAMP enzyme immunoassay kit (Sigma-Aldrich) after the acetylation of each sample.

[^{3}H]Thymidine incorporation assay

H295R cells (30×10^3/well) were precultured in 12-well human fibronectin-coated plates (Biocoat, BD-Falcon) containing 1 ml culture medium. After 48 h, medium was replaced with fresh medium containing 0.3% NuSerum and indicated growth factors were added. After 24 h, 0.5 μCi/well of [methyl-^{3}H]thymidine (Amersham Pharmacia, Piscataway, NJ) was added and incubated for 3 h at 37 C. Cells were then washed with PBS, incubated with 10% ice-cold trichloroacetic acid for 30 min at 4 C, and solubilized in 0.5 M NaOH, and radioactivity was determined by liquid scintillation counter (TRI-CARB 2300TR, Packard Co., Meriden, CT).

Statistical analysis

All results are shown as mean ± SEM. Differences between groups were analyzed for statistical significance using ANOVA (StatView 5.0 software, Abacus Concepts, Inc., Berkeley, CA). P values < 0.05 were accepted as statistically significant.

Results

First, to demonstrate the existence of activin and/or BMP systems in H295R cells, mRNA expression of receptors and ligands composing the activin/BMP system was examined. ALK-2, -3, and -4 but not ALK-6, ActR-II and BMPR-II, and follistatin were clearly expressed in H295R cells (Fig. 1A). As for the activin/BMP ligands, BMP-6 and activin βA and βB subunits were expressed, but BMP-4, -7, and -15 were not detected in this cell line (Fig. 1B). To know whether human adrenal tissues express the activin/BMP system, the receptor subunits and follistatin expressions were examined. As shown in Fig. 1C, normal human adrenal tissue, Aldo adenoma (primary aldosteronism) and cortisol adenoma (Cushing's syndrome) expressed almost all the receptor subunits for activins or BMPs. Notably, the normal adrenal tissue lacked follistatin expression, cortisol adenoma lacked ALK-4, and Aldo adenoma lacked ALK-6 expression, the latter of which shows the identical pattern of receptor expression to the H295R cell line. Therefore, we attempted to approach the biofunction of activin/BMP systems in the adrenal cortex focusing on the Aldo synthesis by H295R cell.

The effect of activin and BMP-6 on Aldo production, one of the major actions performed in zona glomerulosa, was evaluated in H295R cells. In the present experiments, total

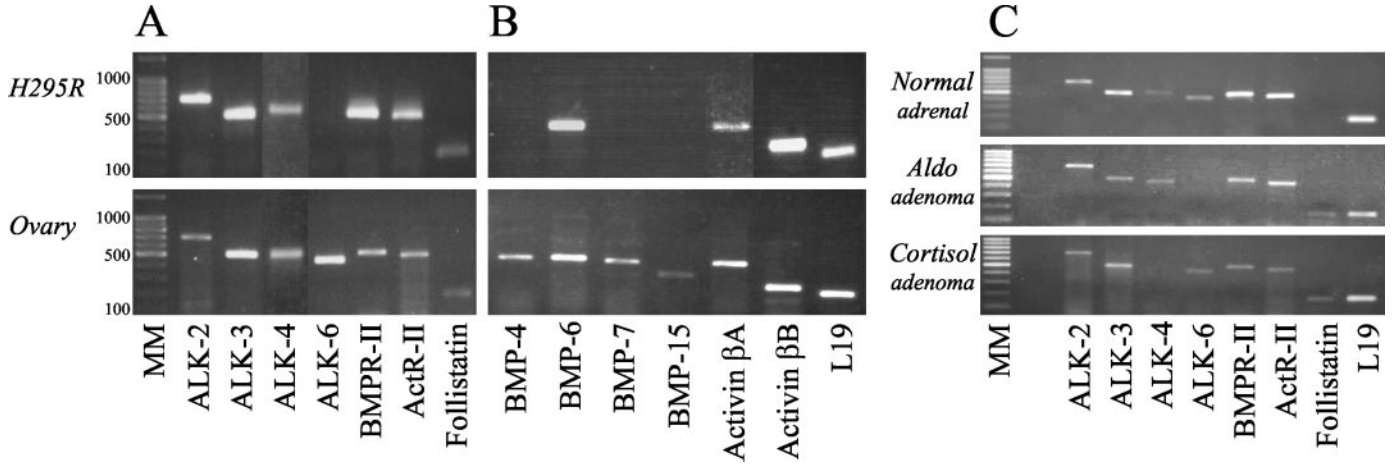

FIG. 1. Expression of activin/BMP system in H295R cells. Total cellular RNAs were extracted from H295R cells. The steady-state levels of mRNAs encoding activin/BMP type-I and -II receptors and follistatin (A) and the ligands including BMP-4, -6, -7, and -15, activin βA and βB, and housekeeping gene L19 (B) were examined by RT-PCR analysis. Expression of activin/BMP receptor subunits and follistatin was examined in the total cellular RNAs extracted from human normal adrenal tissue and tumor tissues of Aldo adenoma and cortisol adenoma by RT-PCR (C). Aliquots of PCR products were electrophoresed on 1.5% agarose gel, visualized by ethidium bromide staining, and shown as representative of those obtained from three independent experiments. MM, Molecular weight marker.

accumulated levels of Aldo for 48 h were determined in the conditioned media. As shown in Fig. 2A, 100 ng/ml activin and BMP-6 exhibited a stimulatory effect on Aldo secretion to levels that were about half of that induced by a PKA activator, BtcAMP (1 mM), or an adenylate cyclase stimulator, FSK (10 μM). The stimulatory effect by activin on Aldo secretion was concentration dependent with an ED_{50} of approximately 30 ng/ml (Fig. 2B), whereas the BMP-6 effect on Aldo production was already saturated at 30 ng/ml (Fig. 2C).

The cellular mechanism by which activin and BMP-6 enhance Aldo production in H295R cells was explored by quantifying mRNA levels of StAR and P450scc, the rate-limiting factors for the early steps of adrenocortical steroidogenesis. As shown in Fig. 3A, the real-time PCR analysis revealed that activin (100 ng/ml) and BMP-6 (100 ng/ml) increased the StAR mRNA level to similar levels as induced by ACTH (100 ng/ml) and Ang II (10 nM) but lower than that induced by BtcAMP (1 mM) and FSK (10 μM). The changes of the StAR mRNA level in each group corresponded to the Aldo levels (compare Figs. 2A and 3A). The level of P450scc mRNA expression was also evaluated, and we found that activin, ACTH, Ang II, BtcAMP, and FSK can enhance the level of P450scc expression in a similar fashion to that of StAR (Fig. 3B). To identify whether the regulation of StAR mRNA expression by BMP-6 and activin occurs at the transcriptional level, the activity of the 5′-promoter region (–1.3 kb) of the StAR gene was evaluated using luciferase reporter assays. As shown in Fig. 4A, both activin and BMP-6 stimulated StAR promoter activity. Activin was more potent than BMP-6. In addition, the late-response genes in the Aldo production, including CYP11B1, which encodes 11β-hydroxylase, and CYP11B2, which encodes P450aldo, were assessed by luciferase assays using H295R cells transfected with reporter plasmids having 5′-promoter regions of CYP11B1 (–1.5 kb) and CYP11B2 (–1.1 kb) genes. In contrast to the response shown in the StAR transcription, BMP-6 was more effective in en-

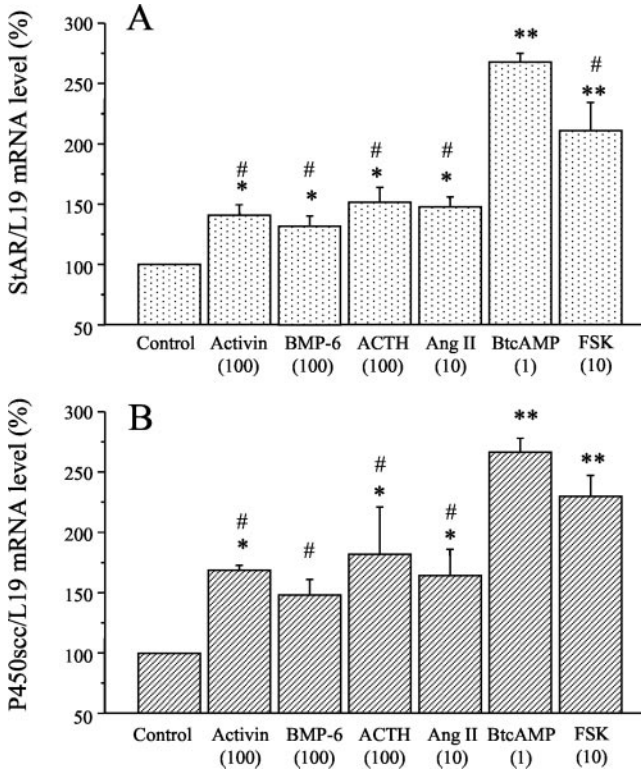

FIG. 3. Effect of activin and BMP-6 on the level of StAR and P450scc mRNA expression in H295R cells. Cells were precultured in 12-well plates (~80% confluency). Culture medium was then replaced with fresh medium containing 0.3% NuSerum with essential supplements and treated for 48 h with activin (100 ng/ml), BMP-6 (100 ng/ml), Ang II (10 nM), ACTH (100 ng/ml), BtcAMP (1 mM), or FSK (10 μM). Total cellular RNA was collected from each well, and the mRNA levels of StAR (A) or P450scc (B) were quantified by real-time RT-PCR analysis and standardized by the level of L19 in each sample. Results show the mean ± SEM of data from three separate experiments, each performed with triplicate treatments; *, $P < 0.05$ and **, $P < 0.01$ *vs.* control; #, $P < 0.01$ *vs.* BtcAMP group.

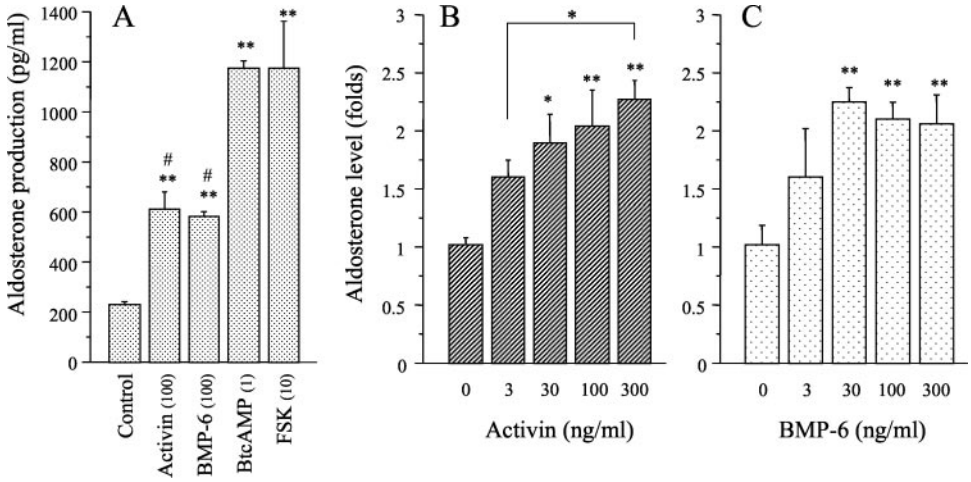

FIG. 2. Effect of Aldo production induced by activin or BMP-6 in H295R cells. Cells were precultured in 24-well plates (~80% confluency), and after 48 h, medium was replaced with fresh medium containing 0.3% NuSerum with essential supplements and then treated for another 48 h with indicated factors. A, The Aldo level was determined by RIA in the conditioned media treated with activin (100 ng/ml), BMP-6 (100 ng/ml), BtcAMP (1 mM), or FSK (10 μM). The concentration-responsive effects of activin (0–300 ng/ml) (B) and BMP-6 (0–300 ng/ml) (C) on Aldo production were then evaluated under the same culture conditions. Results show the mean ± SEM of data from four separate experiments, each performed with triplicate incubations; *, $P < 0.05$ and **, $P < 0.01$ *vs.* control or between the indicated groups; #, $P < 0.01$ *vs.* BtcAMP group.

hancing CYP11B1 and CYP11B2 gene transcription than activin (Fig. 4B).

We attempted to identify the existence of BMP and activin signaling pathways in H295R cells using BMP- or TGF-β-specific reporter assays. As shown in Fig. 5, BMP-6 stimulated the BMP-responsive reporter, Tlx2-Luc (36), suggesting the presence of BMP-responsive Smad1/5 pathway in H295R cells. The 3TP-Luc reporter construct responds to activin and TGF-β and weakly to BMPs (40), whereas the $(CAGA)^9$-Luc reporter construct shows activin-specific responses in various cell lines (38). Indeed, activin strongly and, with by far lesser extent, BMP-6 increased 3TP-Luc and $(CAGA)^9$-Luc, demonstrating the presence of a functional activin-responsive Smad3 pathway in H295R cells (Fig. 5).

To elucidate how the activin/BMP system is involved in the governance of Aldo production induced by Ang II or ACTH, H295R cells were cotreated with activin and BMP-6 in the presence of either ACTH or Ang II. Activin further

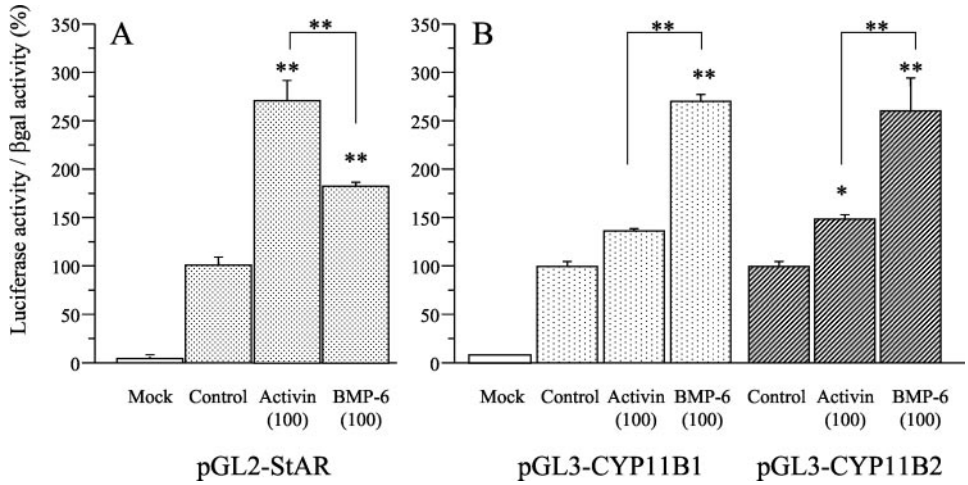

FIG. 4. Transcriptional regulation of StAR, CYP11B1, and CYP11B2 genes in H295R cells. H295R cells were transiently transfected with 1 μg of each luciferase reporter plasmid of StAR (A) or CYP11B1 or CYP11B2 gene (B) and 0.1 μg of pCMV-β-gal for 24 h. The cells were then treated with indicated concentrations of either activin or BMP-6 in the media containing 0.3% NuSerum with essential supplements for 24 h. The cells were washed with PBS and lysed, and the luciferase activity and β-gal activity were measured by luminometer. Results are shown as the ratio of luciferase to β-gal activity and graphed as mean ± SEM of data from three separate experiments, each performed with triplicate treatments; *, $P < 0.05$ and **, $P < 0.01$ *vs.* control or between the indicated groups.

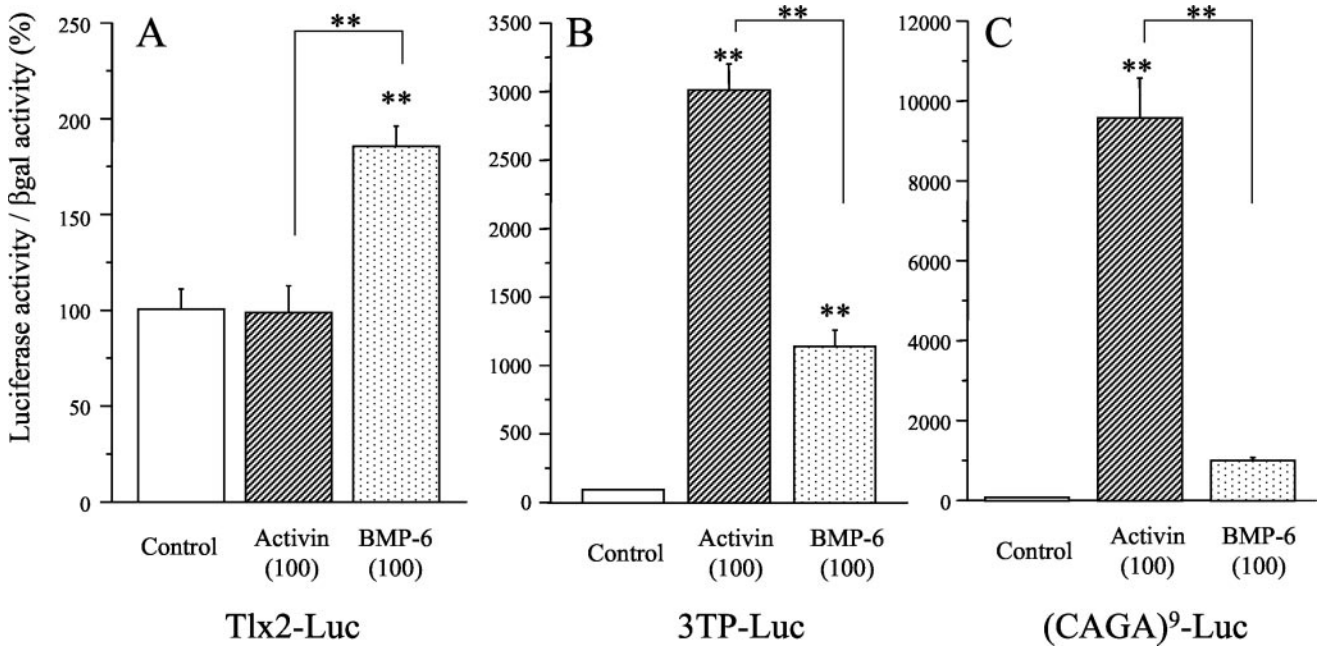

FIG. 5. Effect of activin and BMP-6 on Smad-signaling pathway in H295R cells. H295R cells were transiently transfected with 1 μg of each luciferase reporter plasmid of Tlx2-Luc (A), 3TP-Luc (B), or $(CAGA)^9$-Luc (C) and 0.1 μg of pCMV-β-gal for 24 h. The cells were then treated with indicated concentrations of either activin or BMP-6 in the media containing 0.3% NuSerum with essential supplements for 24 h. The cells were washed with PBS and lysed, and the luciferase activity and β-gal activity were measured by luminometer. Results are shown as the ratio of luciferase to β-gal activity and graphed as mean ± SEM of data from three separate experiments, each performed with triplicate treatments; **, $P < 0.01$ *vs.* control or between the indicated groups.

enhanced Aldo production stimulated by ACTH (100 ng/ml), whereas BMP-6 exhibited no synergistic effect on ACTH-induced Aldo production (Fig. 6A). On the other hand, BMP-6 significantly augmented the Aldo synthesis induced by Ang II (10 nM), although activin did not change the Aldo level induced by Ang II (Fig. 6B). To further assess whether the additive effects of activin on ACTH-induced Aldo synthesis can occur through the second messenger of ACTH signaling, cells were treated with FSK or BtcAMP in a combination with activin or BMP-6. As shown in Fig. 7, activin augmented Aldo production induced by FSK (1 μM) or BtcAMP (0.1 mM), whereas BMP-6 lacked the synergistic effects on either FSK- or BtcAMP-induced Aldo production. Taken together, it seems likely that activin is associated with stimulation of the ACTH-cAMP-PKA pathway for Aldo production, whereas BMP-6 does not affect this pathway.

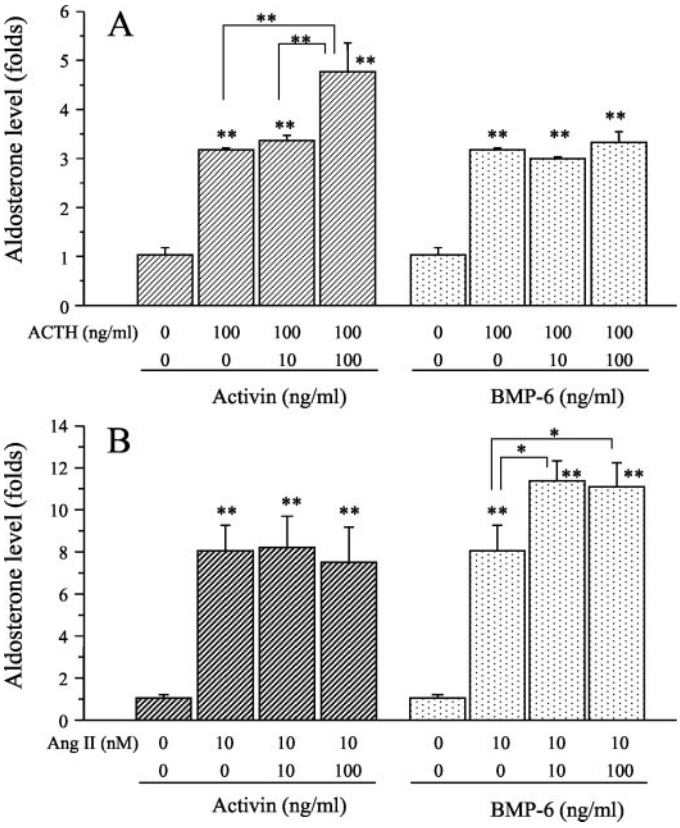

FIG. 6. Effect of activin and BMP-6 on ACTH- or Ang II-induced Aldo production in H295R cells. Cells were precultured in 24-well plates (~80% confluency), and after 48 h, medium was replaced with fresh medium containing 0.3% NuSerum with essential supplements and then treated for 48 h with activin (0–100 ng/ml) or BMP-6 (0–100 ng/ml) in a combination with ACTH (100 ng/ml) (A) or Ang II (10 nM) (B). Results show the mean ± SEM of data from four separate experiments, each performed with triplicate incubations; *, $P < 0.05$ and **, $P < 0.01$ *vs.* control or between the indicated groups.

To further assess the functional link between the activin and ACTH, we directly measured the levels of cAMP synthesis under the presence of activin and BMP-6. To prevent the metabolic effect of phosphodiesterase on cAMP, IBMX was added to the culture medium at 0.1 mM. As shown in Fig. 8, activin (100 ng/ml), ACTH (100 ng/ml), or FSK (10 μM) significantly increased the cAMP production, whereas BMP-6 failed to induce cAMP production in H295R cells. This suggests that both ACTH and activin have stimulatory effects on Aldo production, at least in part, through a common cAMP signaling cascade.

We subsequently attempted to elucidate the endogenous role of activin in Aldo production in H295R cells. Because activin's actions are strictly regulated by its binding protein follistatin, the effect of follistatin on the Aldo production was evaluated in H295R cells. As shown in Fig. 9, follistatin treatment marginally reduced Aldo production and attained maximal suppression to approximately 30% by 200 ng/ml follistatin, suggesting that endogenous activin participates in maintaining basal Aldo secretion by H295R cells. To further pursue the effect of follistatin on ACTH- or Ang II-induced Aldo production, the cells were cotreated with follistatin in the presence of either ACTH or Ang II. Follistatin reduced ACTH-induced Aldo production in a concentration-dependent manner (Fig. 10A). In contrast to the selective abolishment of ACTH-induced Aldo secretion, follistatin did not affect the Aldo levels activated by Ang II treatment (Fig. 10B). Taken together, endogenous activin plays key roles in regulating the Aldo secretion induced by ACTH but not by Ang

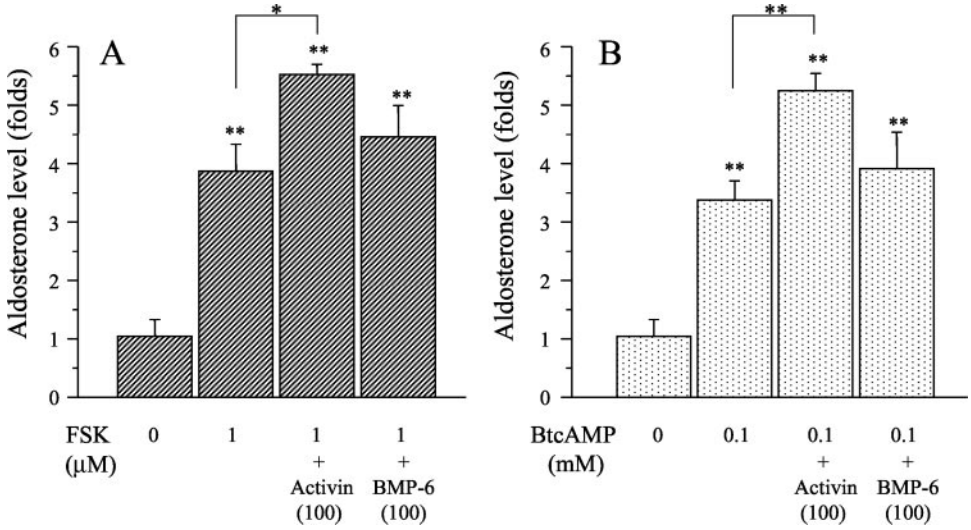

FIG. 7. Effect of activin and BMP-6 on FSK- or BtcAMP-induced Aldo production in H295R cells. Cells were precultured in 24-well plates (~80% confluency), and after 48 h, medium was replaced with fresh medium containing 0.3% NuSerum with essential supplements and then treated for 48 h with activin (100 ng/ml) or BMP-6 (100 ng/ml) in a combination with either FSK (1 μM) (A) or BtcAMP (0.1 mM) (B). Results show the mean ± SEM of data from three separate experiments, each performed with triplicate incubations; *, $P < 0.05$ and **, $P < 0.01$ *vs.* control or between the indicated groups.

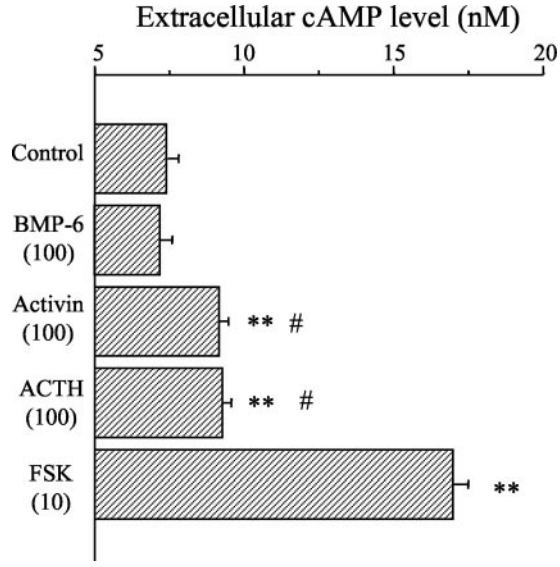

FIG. 8. Effect of activin and BMP-6 on cAMP production in H295R cells. H295R cells (1×10^5 viable cells) were precultured for 24 h, and the medium was replaced with fresh medium containing 0.3% NuSerum with essential supplements and then treated for 48 h with BMP-6 (100 ng/ml), activin (100 ng/ml), ACTH (100 ng/ml), or FSK (10 μM) in the presence of IBMX (0.1 mM). After 48 h, the conditioned medium was collected and the accumulated cAMP levels were determined after acetylation of the samples. Results show the mean ± SEM of data from two separate experiments, each performed with quadruplicate treatments; **, $P < 0.01$ *vs.* control; #, $P < 0.01$ *vs.* FSK group.

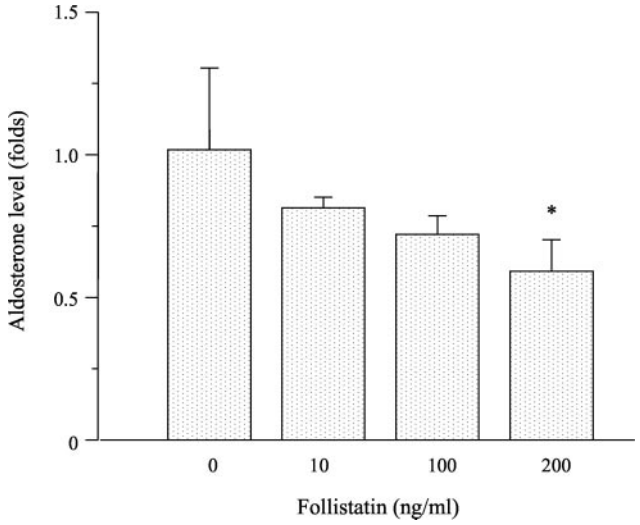

FIG. 9. Effect of follistatin on basal Aldo production in H295R cells. Cells were precultured in 24-well plates (~80% confluency), and after 48 h, medium was replaced with fresh medium containing 0.3% NuSerum with essential supplements and then treated for 48 h with follistatin (0–200 ng/ml). The conditioned media were collected from each well, and Aldo level was measured by RIAs. Results show the mean ± SEM of data from three separate experiments, each performed with triplicate incubations; *, $P < 0.05$ *vs.* control.

II in addition to maintenance of basal Aldo secretion in H295R cells.

Recently, the involvement of MAPK in Ang II signaling has been revealed in Aldo synthesis in adrenocortical cells (8–11). To clarify the involvement of MAPK in activin- or BMP-6-induced Aldo production, H295R cells were treated with a specific ERK inhibitor, U0126 (3 μM) (41, 42), and cocultured in combination with activin, BMP-6, or Ang II. As a result, Ang II-induced Aldo production was significantly decreased by treatment of U0126 (Fig. 11A). The Aldo production induced by activin was not affected by U0126; however, the Aldo level stimulated by BMP-6 was significantly impaired by the pretreatment of U0126 (Fig. 11B). This finding supports our preceding data that BMP-6 but not activin participates in the Ang II-signaling pathway for Aldo production by H295R cells.

Furthermore, the effects of Ang II or ACTH on the expressions of BMP-6 and activin were examined. As for the activin subunits, the expression level of activin βB was relatively higher than that of βA in H295R cells (Fig. 1B). We therefore focused on the expression of activin βB in this study. As shown in Fig. 12, Ang II (10 nM) treatment for 12 h reduced the level of BMP-6 mRNA but increased that of activin βB in H295R cells. However, ACTH (100 ng/ml) treatment did not affect mRNA levels of either BMP-6 or activin βB. Thus, the expression of endogenous activin and BMP-6 is also modulated under the influence of Ang II.

Finally, the impact of the activin/BMP system on the mitogenic property of H295R cells was assessed. As shown in Fig. 13, BMP-6 slightly (~1.8-fold) increased DNA synthesis of H295R cells, but activin exhibited no effect on the DNA synthesis. In contrast, PDGF-BB (100 ng/ml) and TGF-β1 (10–100 ng/ml) showed remarkable increase in DNA synthesis by H295R cells. This result may exclude the possibility that the increase of cell number is the cause of Aldo production induced by either BMP-6 or activin.

Discussion

In the present study we have first clarified the existence of BMP and activin systems composed of ligand subunits (BMP-6 and activin βA/βB), type-I receptors (ALK-2, -3, and -4), type-II receptors (ActR-II and BMPR-II), and the activin/BMP binding protein follistatin in the human adrenocortical cell line H295R. This is the first demonstration regarding the presence of a functional BMP system in a human adrenocortical cell line. Given that human normal adrenal tissue and functioning adrenocortical adenomas obtained from patients with primary aldosteronism or Cushing's syndrome also express a battery of the activin/BMP system, one may consider that an endogenous activin/BMP system is involved in the regulation of adrenocortical function.

Based on our present data, it is most likely that BMP-6 acts through a combination of either of the type-II receptors and ALK-2 in H295R cells, whereas activin can exert its function by binding ActR-II and the subsequent recruitment of ALK-4. Upon binding of BMP ligands to specific type-I and type-II receptors, the receptor complexes cause the phosphorylation of intracellular signaling molecules called Smads, which then translocate to the nucleus and regulate transcription of target genes (26). The receptor-regulated Smads can be grouped into two subsets: Smads 2 and 3 are activated by TGF-β and activin, whereas Smads 1, 5, and 8 are activated by BMPs (27). In the present study, H295R cells were found to possess the active Smad signaling machinery in response to the exoge-

nous activin and BMP-6 based on the findings of reporter gene assays using Tlx2-Luc, 3TP-Luc, and $(CAGA)^9$-Luc plasmids. Although we also examined XVent2-Luc reporter activity, which is known to specifically respond to BMP signaling in several cell lines (42), XVent2 did not react to either BMP-6 or activin (data not shown) in H295R cells, suggesting that these cells lack the cellular machinery for activating the XVent2 gene.

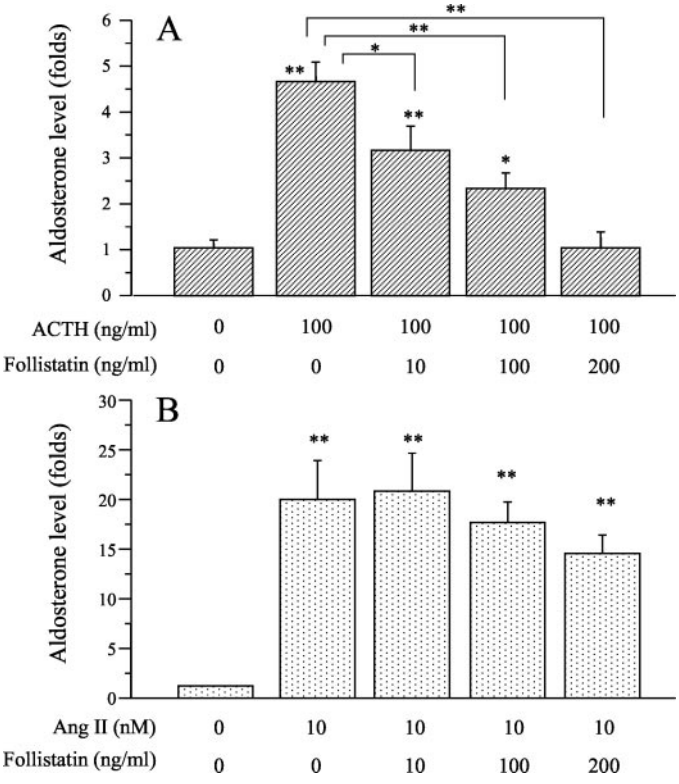

FIG. 10. Effect of follistatin on ACTH- or Ang II-induced Aldo production in H295R cells. Cells were precultured in 24-well plates (~80% confluency), and after 48 h, medium was replaced with fresh medium containing 0.3% NuSerum with essential supplements and then treated for 48 h with follistatin (0–200 ng/ml) in a combination with either ACTH (100 ng/ml) (A) or Ang II (10 nM) (B). Results show the mean ± SEM of data from three separate experiments, each performed with triplicate incubations; *, $P < 0.05$ and **, $P < 0.01$ *vs.* control or between the indicated groups.

The expression of activin/inhibin receptors in the human adrenal gland has been very recently reported by Vanttinen and colleagues (18). They demonstrated the expression of inhibin/activin α, βA, and βB subunits, follistatin, and activin type-I and -II receptor mRNA in adrenocortical cells. In their study, ACTH stimulated the production of both inhibin A and B through PKA signaling and decreased the ratio of activin to inhibin, leading to the hypothesis that a functional activin/inhibin system exists within the human adrenal cortex that acts as a possible autocrine/paracrine regulator of ACTH signaling. However, the regulatory effect of activin on the key functions of adrenocortical cells, *i.e.* steroidogenesis, remains unknown. In our present study, we show that activin and BMP-6 are directly involved in stimulation of Aldo secretion by human adrenocortical cells through different mechanisms involving the ACTH-cAMP-PKA pathway and the Ang II-MAPK pathway, respectively.

Stimulators of Aldo secretion, including Ang II, K^+, and ACTH, act in the early steps of the biosynthetic pathway, *i.e.* before formation of pregnenolone (1, 4). ACTH stimulates the early steps of Aldo biosynthesis, but in long-term experiments it exhibits inhibitory effects on the later steps. In contrast, during long-term experiments, Aldo production seems to be controlled by the regulation of the later stages of the biosynthetic pathway, *i.e.* P450aldo, which controls conversion of DOC to Aldo (43). In the present study, both activin and BMP-6 increased the levels of StAR mRNA in H295R cells, which indicates that both growth factors participate in regulating the rate-limiting step of Aldo synthesis at the early stages of steroidogenesis (44).

Regarding the early steps of Aldo production, the present data implied the existence of a functional link between ACTH and activin in the adrenocortical cells. Activin enhanced ACTH-, FSK-, and BtcAMP-induced Aldo synthesis, and activin directly stimulated cAMP production in H295R cells. Moreover, the finding that ACTH-induced Aldo synthesis is potently suppressed by follistatin, which inhibits activin actions by binding, also supports that activin is a key regulator of ACTH-induced as well as basal Aldo production in this cell line; however, we cannot exclude the possibility that follistatin may inhibit activation of ACTH receptors such as coupling with adenylate cyclase on the cell surface. It is also possible that inhibins induced by ACTH in the adreno-

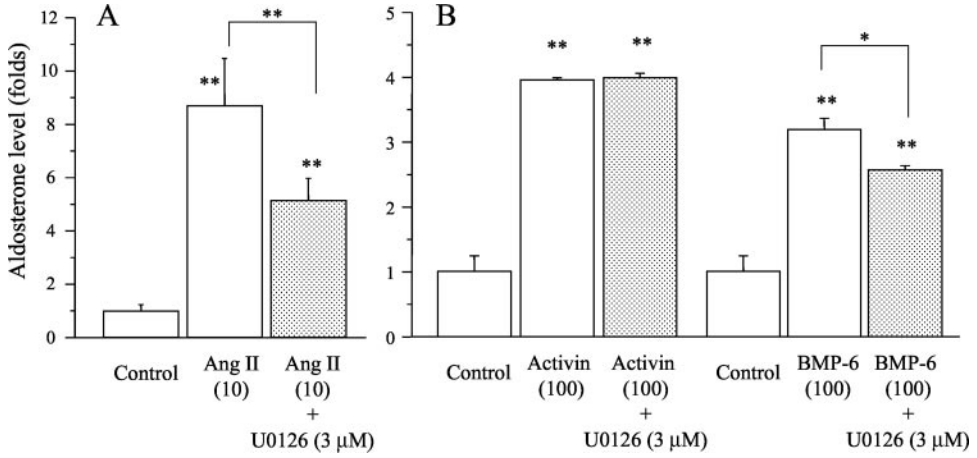

FIG. 11. Effect of ERK inhibition on Aldo production induced by activin or BMP-6 in H295R cells. Cells were precultured in 24-well plates (~80% confluency), and after 48 h, medium was replaced with fresh medium containing 0.3% NuSerum with essential supplements and then treated for 48 h with Ang II (10 nM) (A) or activin (100 ng/ml) or BMP-6 (100 ng/ml) (B) in a combination with U0126 (3 μM). Results show the mean ± SEM of data from three separate experiments, each performed with triplicate incubations; *, $P < 0.05$ and **, $P < 0.01$ *vs.* control or between the indicated groups.

cortical cells (18) may facilitate antagonizing the activin actions in the presence of follistatin. Nevertheless, based on the finding that activin as well as ACTH can commonly stimulate cAMP synthesis in H295R, Aldo production through ACTH is most likely to be supported by the endogenous activins.

After the early phase of adrenal steroidogenesis, pregnenolone is diffused from the inner mitochondrial membrane to the endoplasmic reticulum and converted to progesterone (45). Progesterone is then hydroxylated to DOC, leading to the conversion to the final product Aldo by a mitochondrial enzyme, P450aldo. In rodents and humans, P450aldo is controlled by one gene, CYP11B2, expressed in the zona glomerulosa, whereas a highly homologous gene, CYP11B1, predominantly controls glucocorticoid synthesis in the zona fasciculata (34). Ang II can stimulate transcriptional regulation of the CYP11B2 gene in H295R cells (46) through the AT1 receptor (47), resulting in an increase in CYP11B2 mRNA levels and Aldo production (31, 48). In the present study, using H295R cells, transcription of CYP11B2 was stimulated by activin and strongly stimulated by BMP-6. This differs from the regulation of StAR transcription, which was stimulated more strongly by activin than BMP-6. These results suggest that the involvement of activin and BMP-6 in Aldo synthesis is differential. Namely, activin is primarily involved in the regulation of the early steps of steroidogenesis including StAR and P450scc that are activated by ACTH, whereas BMP-6 is involved in the later steps including CYP11B1 and CYP11B2 that are strongly regulated by Ang II.

In addition to Smad signaling, recent studies have demonstrated that the MAPK family of signaling molecules can modulate the signal transduction of TGF-β superfamily members through cross-talk with the Smad pathway in certain physiological circumstances (49, 50). There is increasing evidence that there can be cross-talk in the BMP signal transduction pathway between the Smads and MAPK family signaling molecules, *i.e.* ERK1/2, p38, and stress-activated protein kinase/Jun N-terminal kinase (49, 50). Recently, the involvement of MAPK of Ang II signaling has been suggested in Aldo synthesis by H295R cells (8–11). In our study, Ang II-induced Aldo production was reduced by a specific ERK inhibitor, U0126, indicating the involvement of ERK in Aldo synthesis in H295R. Importantly, BMP-6-induced, but not activin-induced, Aldo production was reduced by U0126

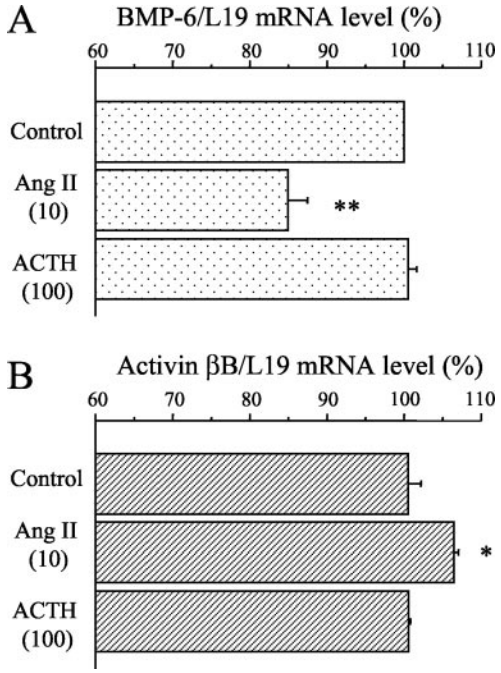

FIG. 12. Effect of Ang II and ACTH on the expression of BMP-6 and activin in H295R cells. Cells were precultured in 12-well plates (~80% confluency). Culture medium was then replaced with fresh medium containing 0.3% NuSerum with essential supplements and treated for 12 h with Ang II (10 nM) or ACTH (100 ng/ml). Total cellular RNA was collected from each well, and the mRNA levels of BMP-6 (A) and activin βB (B) were quantified by real-time RT-PCR analysis and standardized by the level of L19 in each sample. Results show the mean ± SEM of data from three separate experiments, each performed with triplicate treatments; *, $P < 0.05$ and **, $P < 0.01$ *vs.* control.

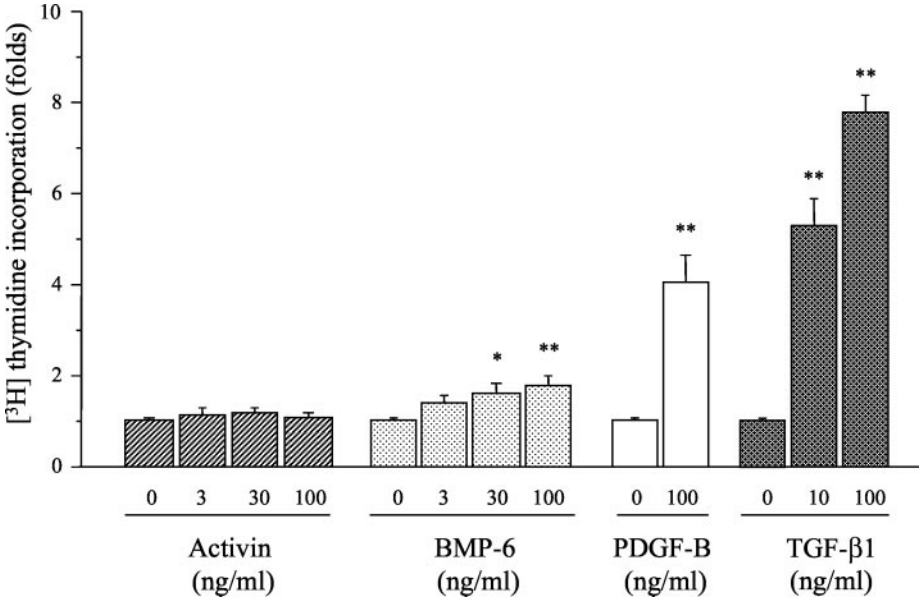

FIG. 13. Effect of activin, BMP-6, PDGF-B, and TGF-β1 on DNA synthesis of H295R cells. Cells (30×10^3/well) were precultured in 12-well plates. Medium was replaced with fresh medium containing 0.3% NuSerum with essential supplements and cultured for another 24 h with indicated growth factors. After 24 h, 0.5 μCi/well of [methyl-^{3}H]thymidine was added and incubated for 3 h at 37 C. Cells were then washed with PBS, incubated with 10% ice-cold trichloroacetic acid, and solubilized in 0.2 M NaOH, and radioactivity was counted. Results show the mean ± SEM of data from three separate experiments, each performed with triplicate incubations; *, $P < 0.05$ and **, $P < 0.01$ *vs.* the control of each group.

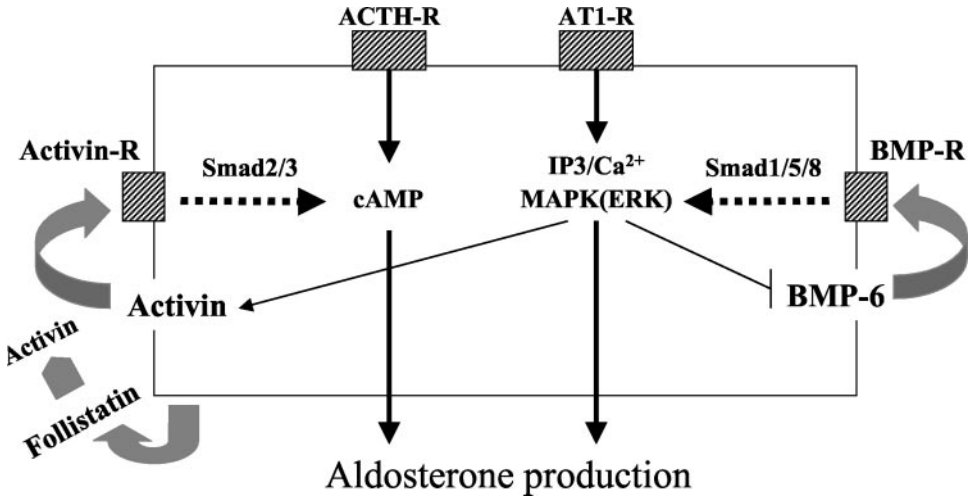

FIG. 14. A possible mechanism by which the activin/BMP system regulates Aldo production in the human adrenocortical cell. Under the functional system for activin and BMPs in human adrenocortical cells, Aldo production induced by activin or BMP-6 is likely to be regulated via the mechanisms that enhance the authentic steroidogenic pathway of either ACTH-cAMP-PKA or Ang II-MAPK, respectively. Ang II action includes inhibition of BMP-6 expression and augmentation of activin expression. R, receptor; IP3, inositol 1,4,5-trisphosphate.

treatment, supporting our hypothesis that BMP-6 but not activin participates in an Ang II-signaling pathway for Aldo production. Gu *et al.* (51) have shown interesting data regarding the functional roles of p38 MAPK in steroidogenesis induced by Ang II or 12-lipoxygenase (12-LO), which is likely to mediate AT1 action in H295R cells. In their study, the 12-LO pathway increased p38 MAPK activity in H295R cells and a p38 specific inhibitor, SB203580, inhibited Ang II- or 12-LO-stimulated cAMP response element-binding protein phosphorylation by half, suggesting the possibility that p38 MAPK activation and cAMP response element-binding protein phosphorylation are involved in Ang II- and 12-LO-induced Aldo production in conjunction with activation of CYP11B2. In this regard, Chabre *et al.* (8) have discussed that MAPK activation is neither necessary nor sufficient for acute stimulation of steroidogenesis, because MAPK activation by basic fibroblast growth factor does increase the cell growth but does not affect steroidogenesis in bovine adrenocortical cells. Additional study is necessary to elucidate how these endogenously expressed activin/BMP systems are associated with these newly identified pathways via MAPK for Aldo synthesis in the adrenal cortex.

Thus, Aldo synthesis and secretion are precisely modulated by the cooperative effects of activin, BMP-6, and follistatin under the major hormonal influence of Ang II and/or ACTH in the adrenocortical cells. In addition, it was revealed that Ang II suppresses the BMP-6 expression but augments the activin expression despite the lack of these modulations by ACTH. Given the fact that BMP-6 preferentially enhances Ang II action for Aldo production whereas Ang II, in turn, diminishes the BMP-6 expression, there could be a negative feedback between Ang II and BMP-6 for controlling the adrenocortical Aldo production. Namely the intraadrenal BMP system may be functionally linked to the systemic renin-angiotensin system. Taking into account that Ang II can stimulate activin expression, Ang II may also play a role in maintaining the ACTH-induced Aldo production through enhancing the activin's effect. The underlying mechanism in the functional link between Ang II and the adrenocortical activin/BMP system needs to be further elucidated.

Collectively, we have demonstrated the existence of functional activin and BMP systems in the human adrenocortical cell line H295R. Although both activin and BMP-6 stimulate Aldo production, activin preferentially enhances the early stages of Aldo synthesis whereas BMP-6 preferentially stimulates the final stages of Aldo steroidogenesis. Consistent with the differential sites of activin and BMP signaling in promoting basal Aldo production, activin selectively augments ACTH-induced Aldo production and BMP-6 selectively augments Ang II-induced Aldo production (Fig. 14), in which BMP-6 and activin expression is further modulated by Ang II. Such an elaborate system composed of endogenous activin/BMP may play crucial roles in adrenal steroidogenesis in an autocrine/paracrine fashion.

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Address all correspondence and requests for reprints to: Fumio Otsuka, M.D., Ph.D., Department of Medicine and Clinical Science, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikata-cho, Okayama City 700-8558, Japan. E-mail: fumiotsu@md.okayama-u.ac.jp.

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