0013-7227/06/$15.00/0
Printed in U.S.A.

Endocrinology 147(6):2681–2689

doi: 10.1210/en.2005-1250

Involvement of Bone Morphogenetic Protein-6 in Differential Regulation of Aldosterone Production by Angiotensin II and Potassium in Human Adrenocortical Cells

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Aldosterone production is modified by several growth factors that reside in the adrenal. We have recently reported the existence of a bone morphogenetic protein (BMP) system in human adrenocortical cells, in which BMP-6 augments aldosterone synthesis. Here, we investigated functional roles of BMP-6, focusing on the differential regulation of aldosterone synthesis induced by angiotensin (Ang) II and potassium (K). In human adrenocortical H295R cells, BMP-6 augmented Ang II-induced CYP11B2 transcription and mRNA and aldosterone production but had no effect on K-induced aldosterone production. Inhibition of endogenous BMP-6 action by neutralizing antibodies impaired aldosterone production induced by Ang II but not that induced by K. Blockage of ligand-receptor binding using extracellular domain (ECD) of BMP type I receptors revealed that ECDs to activin receptor-like kinase (ALK)-2 and ALK-3 significantly reduced the aldosterone production induced by Ang II. None of the type I-receptor ECDs tested had any effect on K-induced aldosterone levels. Overexpression of a dominant negative-activin type II receptor construct selectively decreased Ang II-induced aldosterone production without having any effect on K-induced aldosterone production. BMP type II receptor-dominant negative had no effect on aldosterone induced by either Ang II or K. These results infer that BMP-6 acts through ALK-2, ALK-3, and activin type II receptor receptors in adrenocortical cells. BMP-6 pretreatment extends the induction of ERK1/2 phosphorylation by Ang II and treatment with ECDs to ALK-2 and ALK-3 impaired Ang II-induced ERK phosphorylation. The specific inhibitor of ERK activation, U0126, suppressed the activation of CYP11B2 transcription induced by BMP-6 without affecting Smad phosphorylation and Tlx2-Luc activity. Collectively, the endogenous BMP-6 system plays critical roles in aldosterone production between Ang II and K through ERK signaling pathway. (*Endocrinology* 147: 2681–2689, 2006)

ALDOSTERONE, THE PRINCIPAL mineralocorticoid hormone synthesized in the adrenal cortex, plays crucial roles in the regulation of electrolytes and water balance. A major site of action of aldosterone is the epithelial cell of distal tubules in the kidney, where it promotes sodium (Na) absorption and potassium (K) excretion. Increase in aldosterone production, via alterations in mechanisms controlling its biosynthesis, is associated with Na retention, expansion of plasma volume, and high blood pressure (1, 2). Prolonged elevation of systemic aldosterone levels in turn causes cardiac hypertrophy, fibrosis, and loss of tissue electrolyte balance, which may lead to cardiac dysfunction (3, 4).

Production of aldosterone occurs in the adrenal glomerulosa, which is systemically regulated by angiotensin (Ang) II, K, and ACTH (5, 6). In short-term or acute experiments, all known stimulators of aldosterone secretion including Ang II, K, and ACTH have been shown to act predominantly in the early steps of the biosynthetic pathway, *i.e.* before formation of pregnenolone. In contrast to its stimulatory action in acute studies, in long-term exposure conditions, ACTH rather exerts inhibitory action on the later steps of the steroid biosynthetic pathway, and the major site at which Ang II and K control aldosterone biosynthesis is through control of CYP11B2 encoding P450 aldosterone synthase (P450aldo) (5, 6). Because P450aldo controls the last three steps in the biosynthesis of aldosterone, control of aldosterone production at both early and late stages of the biosynthetic pathway by Ang II and K implies that its production is tightly regulated.

In the presence of these aldosterone stimulators, steroidogenesis in the adrenal cortex is further governed by local autocrine and/or paracrine regulators (7). In this respect, we have recently reported the presence of a functional bone morphogenetic protein (BMP) and activin system complete with ligands including BMP-6 and activin βA/βB; receptors including activin receptor-like kinase (ALK)-2, -3, -4, activin type II receptor (ActRII), and BMP type II receptor (BMPRII); and the binding protein follistatin in the human adrenocortical cell line H295R (8). The receptors for TGF-β superfamily members consist of type I and type II receptors with serine/threonine kinase (9, 10). Activin and TGF-β ligands first bind to the type II receptors followed by the recruitment of type

First Published Online March 9, 2006

Abbreviations: ActRI and ActRII, Activin type I and type II receptor; ALK, activin receptor-like kinase; Ang, angiotensin; AT1R, Ang II type 1 receptor; BMP, bone morphogenetic protein; BMPRI and BMPRII, BMP type I and type II receptor; DN, DN, dominant negative; ECD, extracellular domain; F12, Ham's F-12 medium; β-gal, β-galactosidase; P450aldo, P450 aldosterone synthase; StAR, steroidogenic acute regulatory protein.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

I receptors, whereas BMPs bind type II receptors (BMPRII, ActRII, and ActRIIB), and the appropriate type I receptors act together to form a high-affinity complex. Regarding type I receptors, ALK-2 (also called activin type IA receptor), ALK-3 (BMP type IA receptor), and ALK-6 have been identified as type I receptors for BMPs, whereas ALK-4 is the type I receptor for activin (9, 11).

In H295R cells, BMP-6 caused concentration-dependent increases in aldosterone production with increased expression of rate-limiting factors such as steroidogenic acute regulatory protein (StAR) and P450 steroid side-chain cleavage enzyme (8). BMP-6 enhanced Ang II-induced but not ACTH-induced aldosterone production, whereas activin enhanced ACTH-induced aldosterone production. BMP-6 had no effects on cAMP levels, although activin augmented cAMP production. Follistatin, a binding protein for activin, suppressed basal and ACTH-induced aldosterone but failed to affect Ang II-induced aldosterone levels. Hence, activin acts to regulate adrenal aldosterone synthesis predominantly by modulating the ACTH-cAMP-protein kinase A signaling cascade, whereas BMP-6 is involved in aldosterone production by modulating Ang II signaling in human adrenal cortex (8).

However, many details of the mechanisms by which the endogenous activin/BMP system regulates adrenocortical steroidogenesis remain uncertain. We here investigated the cellular mechanism by which BMP-6 modulates aldosterone synthesis in human adrenocortical cells. The present results implicate a possible role of endogenous BMP-6 in differential regulation of aldosterone production induced by Ang II and K.

Materials and Methods

Reagents and supplies

A 1:1 mixture of DMEM/Ham's F-12 medium (F12), penicillin-streptomycin solution, and Ang II acetate salt were purchased from Sigma-Aldrich Co. Ltd. (St. Louis, MO). Recombinant human TGF-β1 was purchased from PeproTech EC Ltd. (London, UK). NuSerum and insulin-transferrin-sodium selenite Plus (ITS+) were from BD Falcon (Bedford, MA). Recombinant human BMP-6, extracellular domains (ECDs) that lack transmembrane and intracellular domains of ALK-2, ALK-3, and ALK-6 (12), antihuman BMP-6 polyclonal antibody, and normal goat antibody were from R&D Systems (Minneapolis, MN). U0126 and SB203580 were purchased from Promega Corp. (Madison, WI). The plasmid of pGL3-CYP11B2 (13) was kindly provided from Dr. William E. Rainey, Tlx2-Luc (14) was from Dr. Jeff Wrana, and expression plasmids for dominant negative (DN)-BMP receptors including pActRII-DN and pBMPRII-DN were from Dr. Shunichi Shimasaki.

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 4 mM K, 2.5% NuSerum, and 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 (15).

Aldosterone assay

To assess the effect of treatments on aldosterone secretion, monolayered cells (approximately 80% confluency) were precultured in 24-well human fibronectin-coated plates (Biocoat, BD Falcon), and after 48-h culture, the medium was replaced with fresh medium containing 0.3% NuSerum with or without various combinations of Ang II, K, BMP-6, ECDs for ALK-2, -3 and -6, and U0126 or SB203580 at indicated concentrations. H295R cells were then cultured for another 48 h, and the accumulated levels of aldosterone in the conditioned media were determined by RIA using the SPAC-S aldosterone kit (T.F.B. Co., Tokyo, Japan).

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 either alone or combinations of the reagents including Ang II, K, BMP-6, and U0126 or SB203580 at indicated concentrations. After culture for 12 h, the medium was removed, and total cellular RNA was extracted by isothiocyanate-acid phenol-chloroform methods using TRIzol (Invitrogen Corp., Carlsbad, CA), quantified by measuring absorbance at 260 nm, and stored at −80 C until assay. 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. In brief, the extracted RNA (1 μg) was subjected to a RT reaction using the First-Strand cDNA Synthesis System (Invitrogen Corp.) with random hexamer (2 ng/μl), reverse transcriptase (200 U), and deoxynucleotide triphosphate (0.5 mM). PCR product sizes and the primer settings of GenBank accession number were as follows: StAR, 327 bp (U17280: 449–469 and 757–775); CYP11B2, 141 bp (NM_000498: 704–723 and 825–844); and housekeeping gene, ribosomal protein L19, 190 bp (NM_000981, 401–420 and 571–590). For the quantification of StAR, CYP11B2, 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 as we have reported (8). Accumulated levels of fluorescence were analyzed by second derivative method after the melting curve analysis, and then the expression levels of target gene transcripts were standardized by L19 level in each sample.

Transient transfection and luciferase assay

After 24-h preculture in 12-well human fibronectin-coated plates (Biocoat, BD Falcon), H295R cells (approximately 60% confluency) were transiently transfected with 1 μg each luciferase reporter plasmid (pGL3-CYP11B2 and Tlx2-Luc) and 0.1 μg pCMV-β-gal using FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN) for 24 h. The cells were then treated with either alone or combinations of Ang II, K, BMP-6, and U0126 at indicated concentrations 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-galactosidase (β-gal) activity of the cell lysate were measured by luminescencer-PSN (ATTO, Tokyo, Japan) as we have earlier reported (16). The data were shown as the ratio of luciferase to β-gal activity.

DN receptor study

DN receptor study was performed as we have previously reported (17). In brief, ECDs of BMP receptors were independently overexpressed on H295R using expression plasmids to produce DN models of BMP receptors. After 24-h preculture in 12-well human fibronectin-coated plates (Biocoat, BD Falcon), cells (approximately 70% confluency) were transiently transfected with pBUD-CE4.1 (mock), pActRII-DN (containing extracellular and transmembrane domains of ActRII), or pBMPRII-DN (containing extracellular and transmembrane domains of BMPRII) at 0.1 and 0.5 pmol using FuGENE 6 (Roche Molecular Biochemicals) for 24 h. The transfected cells were subsequently cultured in fresh medium containing 0.3% NuSerum with Ang II (10 nM) or K (16 mM) for 48 h, and the conditioned medium was used for measurement for aldosterone as mentioned above.

SDS-PAGE and immunoblotting analysis

Cells (1×10^6 viable cells) were seeded in 12-well human fibronectin-coated plates (Biocoat, BD Falcon) in DMEM/F12 containing 0.3%

NuSerum. After 24-h preculture, indicated concentrations of BMP-6 and either Ang II or K were added to the culture media in combinations of ECDs (for ALK-2, -3, and -6) or MAPK inhibitors (U0126 and SB203580). After stimulation by hormones and growth factors in the indicated time course, cells were solubilized in 100 μl radioimmunoprecipitation assay lysis buffer (Upstate Biotechnology, Charlottesville, VA) containing 1 mM Na_3VO_4, 1 mM NaF, 2% SDS, and 4% β-mercaptoethanol as we have earlier reported (16). The cell lysates were then subjected to SDS-PAGE immunoblotting analysis using antiphospho- and antitotal-p38 MAPK antibodies (Cell Signaling Technology, Inc., Beverly, MA), antiphospho- and antitotal-ERK1/2 MAP kinases antibodies (Cell Signaling Technology, Inc.), antiphospho-Smad1/5/8 antibody (Cell Signaling Technology, Inc.), antihuman BMP-6 antibody (R&D Systems), and antiactin antibody (Sigma-Aldrich Co. Ltd.). The relative integrated density of each protein band was digitized by NIH image J 1.34s (National Institutes of Health, Bethesda, MD).

Statistical analysis

All results are shown as mean ± SEM 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 test (StatView 5.0 software, Abacus Concepts, Inc., Berkeley, CA). $P < 0.05$ was accepted as statistically significant.

Results

First, to compare the effect of BMP-6 on aldosterone synthesis induced by Ang II with that induced by K, the transcriptional regulation of CYP11B2 (encoding P450aldo) in H295R cells was monitored together with the levels of aldosterone that accumulated in the culture medium. Transcriptional regulation of CYP11B2 was analyzed by luciferase assays using H295R cells transiently transfected with a reporter construct using the 5′-promoter region (−1521 bp) of the CYP11B2 gene. We have previously reported that BMP-6 stimulates CYP11B2 transcription and aldosterone production to approximately 2-fold in H295R cells (8). As shown in Fig. 1A, Ang II and K stimulated CYP11B2 transcriptional activity in a concentration-dependent manner. The actions of Ang II saturated at 10 nM and K action saturated at 28 mM. Notably, BMP-6 significantly enhanced Ang II (10 nM)-induced CYP11B2 transcription but had no effect on K (16 mM)-induced CYP11B2 transcription (Fig. 1B). Steady-state mRNA levels were also examined by quantitative real-time PCR analysis. CYP11B2 mRNA levels were increased to approximately 8- and approximately 6-fold by Ang II (10 nM) and K (16 mM) stimulation for 12 h, respectively (Fig. 1C). Consistent with the CYP11B2 promoter assay data, BMP-6 significantly enhanced CYP11B2 mRNA levels induced by Ang II but not that induced by K (Fig. 1C). Furthermore, BMP-6 significantly increased aldosterone production induced by Ang II for 48-h culture but not that by K (Fig. 1D). Thus, BMP-6 specifically augmented Ang II-induced activation of aldosterone synthesis.

BMP-6 protein migrating as 18 kDa was detected in the cell lysates in H295R cells cultured for 24–72 h, and BMP-6 protein was also detected in a concentrated fraction of the conditioned medium (data not shown). To characterize the roles of endogenous BMP-6, we investigated whether the action of BMP-6 would be perturbed by pretreating cells with a BMP-6 neutralizing antibody (anti-BMP-6 IgG) or with a control nonspecific antibody (control IgG). As shown in Fig. 2A, the pretreatment of anti-BMP-6 IgG caused a concentration-

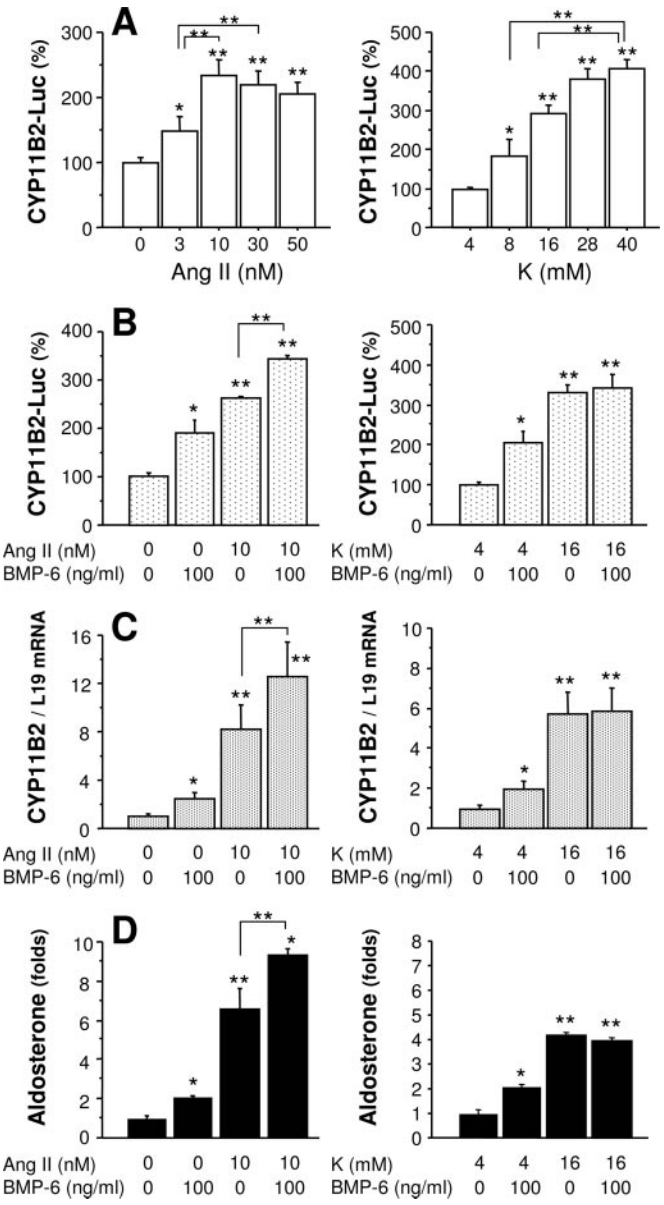

FIG. 1. Effects of BMP-6 on Ang II- or K-induced aldosterone synthesis by H295R cells. A, Concentration-dependent effects of Ang II and K on CYP11B2 transcription. B, Transcriptional regulation of CYP11B2 by BMP-6. Cells were transiently transfected with 1 μg pGL3-CYP11B2 and 0.1 μg pCMV-β-gal for 24 h. The cells were then replaced with fresh media containing 0.3% NuSerum and treated with indicated concentrations of Ang II (nanomolar) or K (millimolar) in the absence or presence of BMP-6 (nanograms per milliliter) 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. C, Effects of BMP-6 on Ang II- or K-induced CYP11B2 mRNA level. After preculture, cells were cultured in 12-well plates with either Ang II or K in combination with BMP-6 for 12 h. Total cellular RNA was collected, and the CYP11B2 mRNA levels were quantified by real-time PCR analysis being standardized by the level of a housekeeping gene, ribosomal protein L19 in each sample. D, Effects of BMP-6 on Ang II- or K-induced aldosterone production. After preculture of the cells, the medium was replaced with fresh medium containing 0.3% NuSerum and treated with either Ang II or K in combination with BMP-6 for 48 h. Accumulated aldosterone levels in the conditioned media were determined by RIA. All results are shown as mean ± SEM of data from at least three separate experiments, each performed with triplicate samples. *, $P < 0.05$; **, $P < 0.01$ *vs.* control or between the indicated groups.

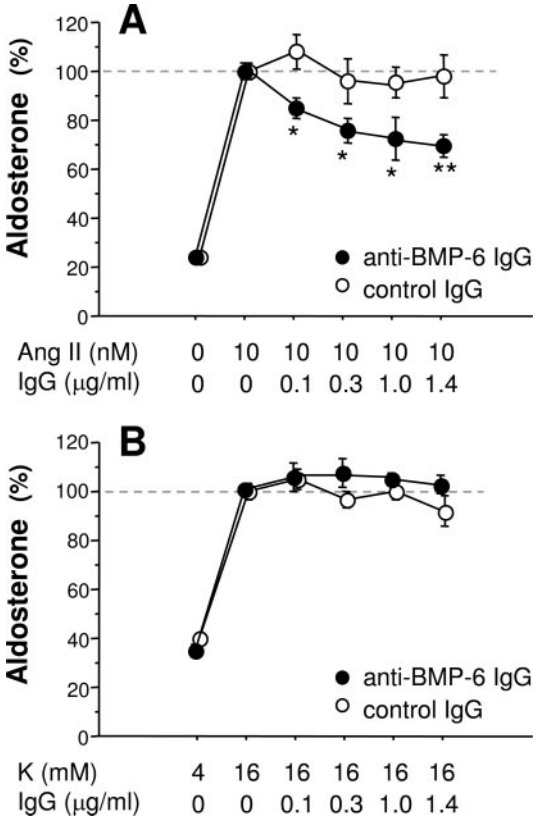

FIG. 2. Neutralizing effects of BMP-6 antibody on Ang II- and K-induced aldosterone production by H295R cells. After 48-h preculture, medium was replaced with fresh medium containing 0.3% NuSerum with essential supplements and then treated for another 48 h with anti-BMP-6 IgG or control IgG (0.1–1.4 μg/ml) with indicated concentrations of Ang II (nanomolar) (A) or K (millimolar) (B). The aldosterone levels were determined by RIA in the conditioned media. All results are shown as mean ± SEM of data from at least three separate experiments, each performed with triplicate samples. *, $P < 0.05$; **, $P < 0.01$ *vs.* control IgG group.

dependent reduction of Ang II-induced aldosterone production by 30%. Under the same conditions, control IgG had no effect. In contrast, K-induced aldosterone production was suppressed by neither anti-BMP-6 IgG nor control IgG (Fig. 2B). This result indicated that endogenous BMP-6 plays a crucial role in enhancement of aldosterone production specifically induced by Ang II but not that induced by K, a finding consistent with the results of experiments performed with exogenously added BMP-6 (see Fig. 1D).

We next investigated the roles of endogenous BMP-6 in aldosterone production by inhibiting its receptor binding using various ECDs of BMP type I receptors. H295R cells were cocultured with ECD proteins of ALK-2, -3, and -6 in the presence of Ang II or K. As shown in Fig. 3, aldosterone levels stimulated by Ang II were significantly suppressed by ALK-2-ECD and ALK-3-ECD for 48-h culture, whereas K-induced aldosterone production was not affected by the ECD treatments. Given our previously reported finding that H295R cells express ALK-2 and -3 but not -6 (8), BMP-6 seems likely to elicit crucial roles in regulation of Ang II-induced aldosterone, possibly through ALK-2 and ALK-3.

Involvement of type II receptors including ActRII and BMPRII was also studied by overexpressing DN constructs of ActRII-DN and BMPRII-DN in H295R cells. The DN constructs are designed to express only the extracellular ligand-binding region and transmembrane domain of the respective receptors (17); thus, they act in a DN fashion by sequestering the ligands, preventing binding to endogenous receptors. Expression of type II receptor DN constructs in H295R cells reduced mRNA levels of Id-1, a common target gene of BMP signaling, by approximately 35% in the presence of 3 nM BMP ligands (data not shown). In this study, Ang II-induced aldosterone levels for 48 h were specifically suppressed by overexpression of ActRII-DN but not by BMPRII-DN (Fig. 4A). K-induced aldosterone levels were not altered by either of the type II receptor DN constructs (Fig. 4B).

The significance of MAPK in Ang II signaling has been recently recognized in regulating aldosterone production by adrenocortical cells (18–22). We also earlier reported that Ang II-induced aldosterone production is inhibited by a specific ERK inhibitor U0126 in H295R cells (8). To elucidate intracellular signal transduction mechanisms involved in the BMP-6 activation of aldosterone production, we first examined whether activation of MAPK signaling molecules correlates with aldosterone production. As shown in Fig. 5A, *left*, Western immunoblots using antiphospho-ERK1/2 and antiphospho-p38 antibodies revealed that treatment with

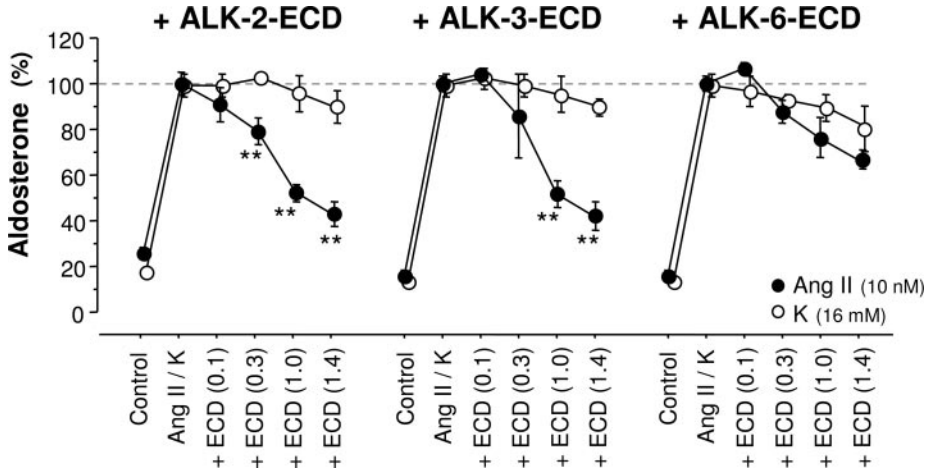

FIG. 3. Inhibitory effects of BMP type I receptor ECDs on Ang II- and K-induced aldosterone production in H295R cells. Cells were precultured in 24-well plates, and the medium was replaced with fresh medium containing 0.3% NuSerum. The cells were then cultured with ECD proteins of ALK-2, -3, or -6 (0.1–1.4 μg/ml) in a combination with Ang II (10 nM) and K (16 mM) for 48 h. The aldosterone levels were determined by RIA in the conditioned media. All results are shown as mean ± SEM of data from at least three separate experiments, each performed with triplicate samples. **, $P < 0.01$ *vs.* K-treated group.

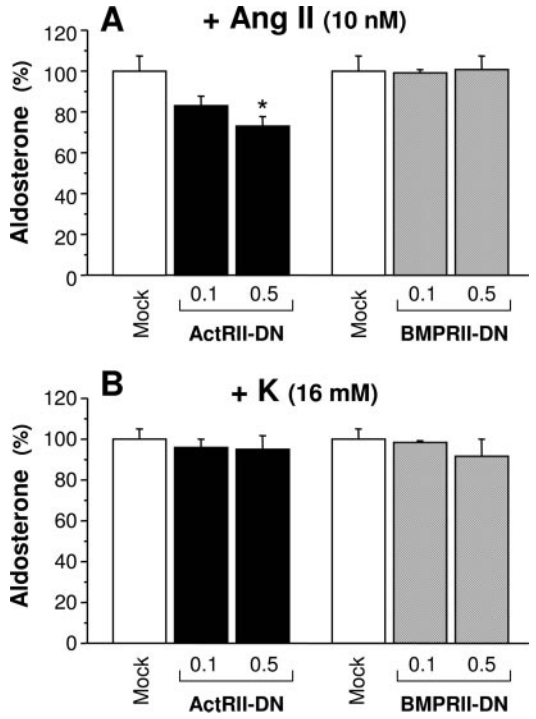

FIG. 4. DN effects of BMP type II receptors on Ang II- and K-induced aldosterone production by H295R cells. After 24-h preculture in 12-well plates, cells were transiently transfected with pBud-CE4.1 (Mock), pActRII-DN, and pBMPRII-DN at 0.1 and 0.5 pmol for 24 h to produce DNs of BMP type II receptors. The transfected cells were subsequently cultured with Ang II (10 nM) (A) or K (16 mM) (B) for 48 h. The aldosterone levels were determined by RIA in the conditioned media. All results are shown as mean ± SEM of data from at least three separate experiments, each performed with triplicate samples. *, $P < 0.05$ *vs.* mock control.

Ang II for 15 min potently induced ERK and p38 phosphorylation. In contrast, under the same conditions, K was relatively ineffective in stimulating phosphorylation of these MAPK molecules. As seen in Fig. 5A, *right*, the effects of MAPK inhibitors were monitored by immunoblotting analysis, showing that SB203580 (1 μM) and U0126 (3 μM) specifically inhibit Ang II-induced phosphorylation of p38 and ERK, respectively, in H295R cells. Notably, the inhibition of ERK activation by U0126 abolished Ang II-induced aldosterone production, but the inhibition of p38 activation by SB203580 had no effect (Fig. 5B). K-induced aldosterone production was not affected either by U0126 or SB203580. This suggests that ERK activation plays a key role in Ang II- but not K-induced aldosterone production. Activation of p38 likely plays at most a very minor role in aldosterone production by Ang II and K. The inhibitory effects of MAPK inhibitors on aldosterone production were accompanied by the decreases in mRNA levels of StAR as well as CYP11B2 (Fig. 5B).

To elucidate the mechanism whereby BMP-6 is involved in regulation of ERK phosphorylation induced by Ang II, H295R cells were treated with BMP-6 in the presence or absence of Ang II or K. As shown in Fig. 6A, ERK1/2 phosphorylation was potently stimulated by Ang II. K treatment marginally and transiently stimulated ERK1/2 for a period 5–15 min after administration. BMP-6 alone had no effects on ERK activation, and BMP-6 did not elicit apparent additive effects on Ang II-induced ERK phosphorylation at acute exposure. However, under longer stimulation with Ang II and BMP-6 for 2–4 h, BMP-6 exerted significantly additive effects on the ERK phosphorylation induced by Ang II (Fig. 6B). It is of note that 1-h pretreatment with BMP-6 efficaciously enhanced the ERK phosphorylation induced by Ang II during 1- to 4-h culture conditions (Fig. 6C). To confirm whether the effects of BMP-6 on ERK activation are related to the BMP receptor binding, we tested the cells with ECDs of BMP type I receptors, with the result that 1-h pretreatment with ALK-2-ECD, and less effectively ALK-3-ECD, exhibited inhibitory effects on the acute ERK activation (15 min) induced by Ang II (Fig. 7). In a later phase (2 h), pretreatment with ALK-3-ECD also significantly suppressed the ERK activation induced by Ang II, and it attained to the same reduction as the effect of ALK-2-ECD (Fig. 7). It is therefore concluded that BMP-6 is most likely to play key roles in enhancing and sustaining the ERK activation induced by Ang II via ALK-2 and/or ALK-3 on H295R cells.

The interaction between BMP-Smad signaling and ERK activation was assessed. As seen in Fig. 8A, BMP-6 clearly induced Smad1/5/8 phosphorylation, and the effects were not altered under the presence of Ang II and K. Specificity of the Smad activation effect was confirmed by the relative lack of Smad1/5/8 phosphorylation induced by TGF-β1. The activation of phospho-Smad1/5/8 by BMP-6 was not altered by treatment of an ERK inhibitor U0126. Consistent with this effect, U0126 did not affect the BMP-responsive reporter activity evaluated by Tlx2-Luc assay (Fig. 8B) [the *Tlx-2* homeobox gene is expressed in the primitive streak of mouse embryos and found to be a downstream target gene for BMP signaling (14)]. However, BMP-6-induced CYP11B2-Luc activation was suppressed by U0126 concentration dependently (Fig. 8C). Thus, the inhibition of ERK using U0126 is effective for inhibiting CYP11B2 transcriptional regulation induced by BMP-6, whereas it did not affect BMP-6-induced Smad1/5/8 signal activation, leading to the possibility that ERK activation contributes to aldosterone production at the downstream of Smad activation.

Discussion

In the present study, we elucidated the mechanism by which BMP-6 controls aldosterone production including the identification of BMP-6 signaling pathways and possible receptors in H295R cells. As we previously reported, administration of BMP-6 to H295R cells causes concentration-dependent increases in aldosterone production as well as increased transcription of StAR and CYP11B2 in the absence of Ang II (8). In the present study, we uncovered a mechanism by which BMP-6 specifically augments transcriptional activation and mRNA levels of CYP11B2 and aldosterone production induced by Ang II. In contrast to its effects on Ang II action, BMP-6 had no effect on the actions of K. Based on our finding that inhibition of endogenous BMP-6 by neutralizing antibodies reduced Ang II- but not K-induced aldosterone production, it is likely that endogenous BMP-6 produced by H295R cells plays an important autocrine role in regulating the steroidogenic actions of Ang II.

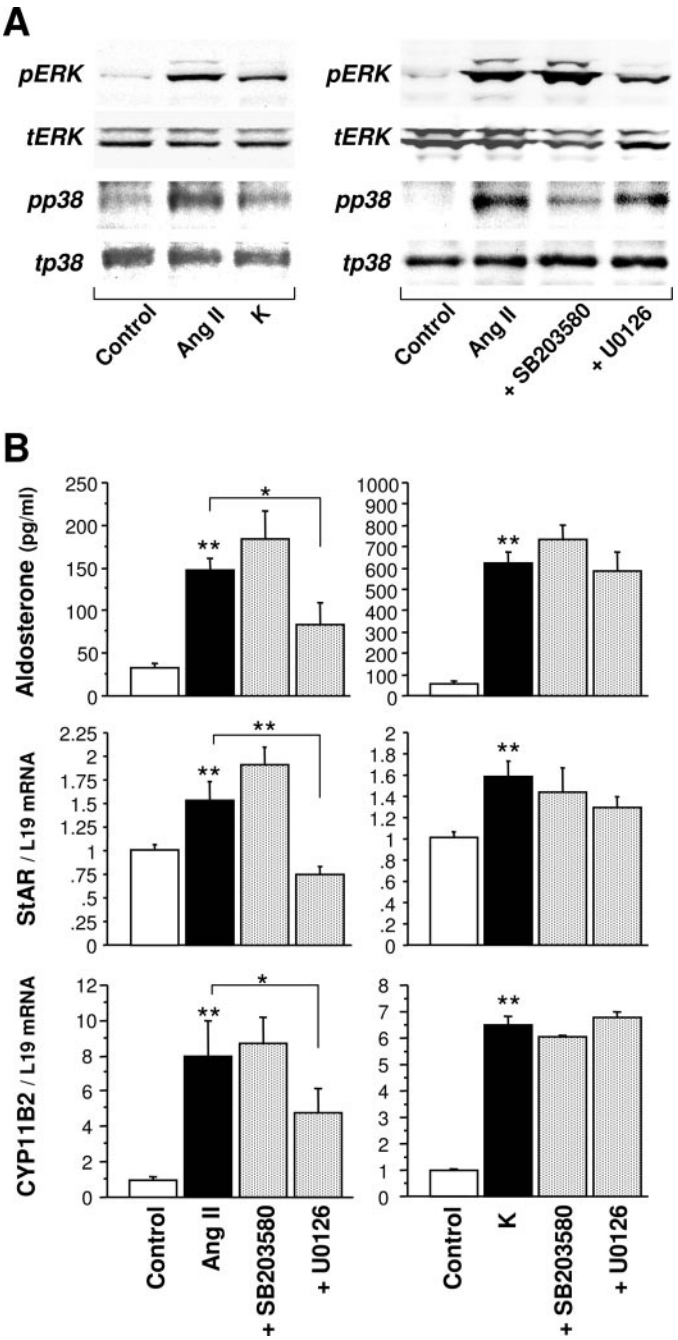

FIG. 5. Effects of Ang II- and K-induced MAPK activation on aldosterone production by H295R cells. A, Effects of Ang II, K, and MAPK inhibitors on ERK1/2 and p38 phosphorylation. Cells were precultured in 12-well plates, and the medium was replaced with fresh medium containing 0.3% NuSerum. The cells were then stimulated with Ang II (10 nM) and K (16 mM) for 15 min in the presence or absence of a p38 inhibitor SB203580 (1 μM) and an ERK inhibitor U0126 (3 μM). The cell lysates were subjected to immunoblotting analysis using antiphospho-ERK1/2 (pERK), antitotal-ERK1/2 (tERK), antiphospho-p38 (pp38), and antitotal-p38 (tp38) antibodies. Results were shown as representative of those obtained from three independent experiments. B, Effects of MAPK inhibitors on aldosterone production and mRNA levels of StAR and CYP11B2. For aldosterone assay, cells were treated with fresh medium containing 0.3% NuSerum with SB203580 (1 μM) or U0126 (3 μM) in a combination with Ang II (10 nM) or K (16 mM) for 48 h. The aldosterone levels were determined by RIA in the conditioned media. To examine the effects of MAPK inhibitors on StAR and CYP11B2 mRNA levels, cells were precultured in six-well plates and treated with SB203580 (1 μM) or U0126 (3 μM) in a combination with Ang II (10 nM) or K (16 mM) for 12 h. Total cellular RNA was collected, and the mRNA levels of StAR and CYP11B2 were quantified by real-time PCR analysis and standardized by the level of L19 in each sample. All results are shown as mean ± SEM of data from at least three separate experiments, each performed with triplicate samples. *, $P < 0.05$; **, $P < 0.01$ *vs.* control or between the indicated groups.

Perturbation of BMP receptor binding using type I receptor-ECDs and DN constructs of type II receptors potently reduced aldosterone production only when stimulated by Ang II but not that stimulated by K. Based on these findings, the likely BMP-6 receptor combinations in H295R cells are ALK-2/ActRII and ALK-3/ActRII. BMP-6 is known to readily bind ALK-2 and ALK-6, although there is considerable promiscuity or cross-reactivity between BMP ligands, including BMP-6 and various type I receptors (23, 24). Because H295R cells do not express ALK-6 (8), we postulate that ALK-2 and/or ALK-3 are likely principal BMP type I receptors for BMP-6 in this cell line.

Upon binding of BMP ligands to specific type I and II receptors, the receptor complexes cause the phosphorylation of intracellular signaling Smad molecules, which then translocate to the nucleus and regulate transcription of target genes (25). The receptor-regulated Smads can be grouped into two subsets: Smad2/3, which are activated by TGF-β and activin; and Smad1/5/8, which are activated by BMPs (10). In the present study, activated Smad1/5/8 signaling was clearly elicited by BMP-6 in H295R cells regardless of the presence or absence of Ang II and K.

Ang II and K stimulate transcriptional regulation of the CYP11B2 gene in H295R cells (26), which in turn results in increases in CYP11B2 mRNA levels and aldosterone production (15, 27). The Ang II action in aldosterone synthesis is solely mediated by Ang II type 1 receptor (AT1R) (28) because Ang II type 2 receptor is not expressed in H295R cells. One of the common regulatory factors of AT1R signaling and K in regulation of CYP11B2 is intracellular calcium (Ca^{2+}) influx in adrenocortical cells (5, 29). Ang II elevates cytosolic Ca^{2+} at the expense of internal and external Ca^{2+} compartments, whereas K increases cytosolic Ca^{2+} concentration at the expense of the external space stores (29–31). Involvement of BMP-6 in regulation of Ca^{2+} influx by Ang II and K need to be elucidated in future studies.

In an attempt to identify essential components of the complex network involved in the regulation Ang II- and K-induced aldosterone production, we focused on the interaction between BMP signaling and MAPK pathways. 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 (32, 33). 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 (32, 33).

To uncover the mechanism by which the BMP-6 system activates Ang II-induced aldosterone production, we examined significance of MAPK activation with regard to aldo-

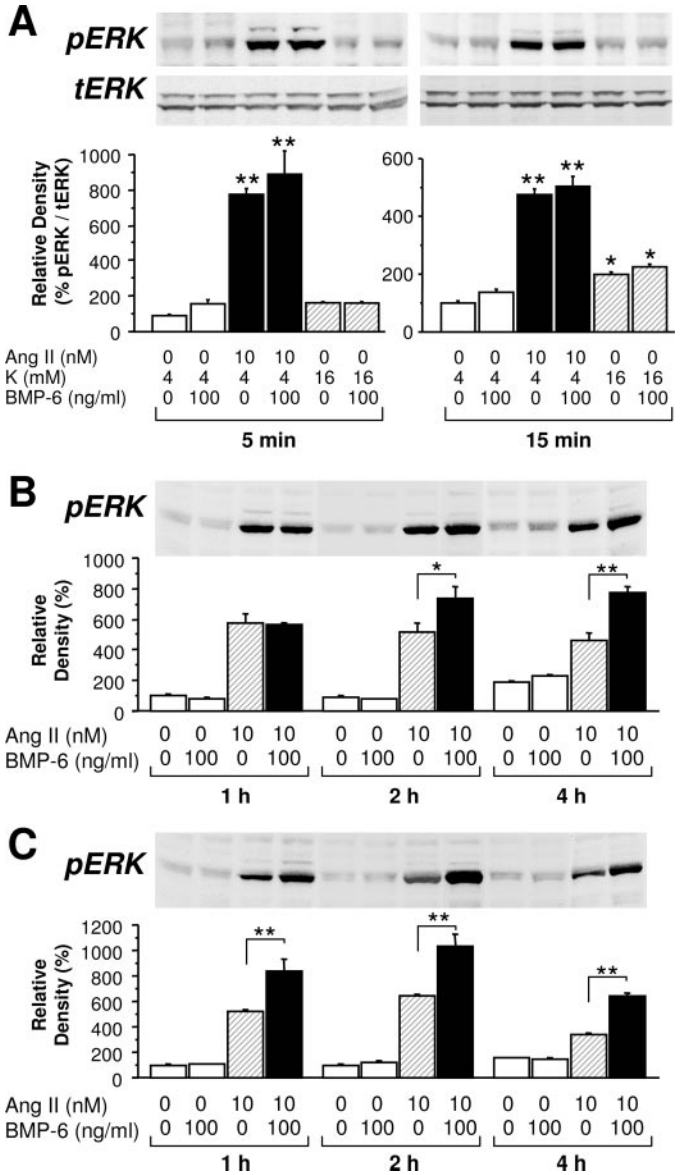

FIG. 6. Effects of BMP-6 on Ang II-induced ERK1/2 phosphorylation in H295R cells. A, Acute effects of BMP-6 on ERK phosphorylation. Cells were precultured in 12-well plates with fresh medium containing 0.3% NuSerum and treated with indicated concentrations of BMP-6 (nanograms per milliliter) in the presence or absence of Ang II (nanomolar) and K (millimolar) for 5 and 15 min. B, Long-term effects of BMP-6 on ERK phosphorylation. After preculture, the cells were treated with BMP-6 (nanograms per milliliter) in the presence or absence of Ang II (nanomolar) for 1, 2, and 4 h. C, Effects of BMP-6 pretreatment on ERK phosphorylation. After preculture, cells were preincubated with BMP-6 (nanograms per milliliter) for 1 h and then stimulated with Ang II (nanomolar) for 1, 2, and 4 h. The cell lysates collected in each experiment were subjected to immunoblotting analysis using antiphospho-ERK1/2 (pERK) and antitotal-ERK1/2 (tERK) antibodies. The band intensity of scanned images was statistically analyzed. All results are shown as mean ± SEM of data from at least three separate experiments, each performed with triplicate samples. *, $P < 0.05$; **, $P < 0.01$ *vs.* each control or between the indicated groups.

sterone production. The involvement of MAPK of Ang II signaling in the regulation of aldosterone synthesis by adrenocortical cells, including H295R cells, has been recently suggested (8, 18–22). In the present study, Ang II potently

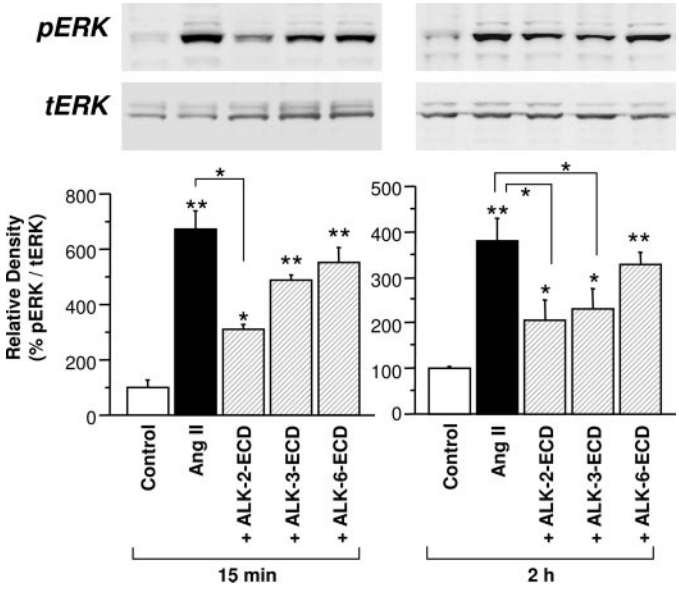

FIG. 7. Effects of BMP type I receptor blockage on Ang II-induced ERK1/2 phosphorylation in H295R cells. Cells were precultured in 12-well plates with fresh medium containing 0.3% NuSerum for 12 h. The cells were pretreated with ECDs for ALK-2, -3, and -6 (1.0 μg/ml) for 1 h and then stimulated with Ang II (10 nM) for 15 min and 2 h. The cell lysates were subjected to immunoblotting analysis using antiphospho-ERK1/2 (pERK) and antitotal-ERK1/2 (tERK) antibodies. The band intensity of scanned images was statistically analyzed. All results are shown as mean ± SEM of data from at least three separate experiments, each performed with triplicate samples. *, $P < 0.05$; **, $P < 0.01$ *vs.* control or between the indicated groups.

induced ERK and p38 phosphorylation. Yet, the chemical inhibition of ERK and p38 activation by U0126 and SB203580, respectively, demonstrated that Ang II-induced aldosterone was abolished by U0126 but not by SB203580. Aldosterone production induced by K was not affected by either U0126 or SB203580. This suggests that ERK activation plays a key role in Ang II- but not K-induced aldosterone production, whereas p38 MAPK activation is not significantly involved in modulating aldosterone production induced by either Ang II or K.

BMP-6 also governed the activation of ERK phosphorylation induced by Ang II (Fig. 9). Previous studies have shown that the maximal activation of ERK signaling in adrenocortical cells is achieved after short-term exposure to Ang II and peaks at around 30 min (20, 34–36). Based on experiments using primary bovine adrenocortical cell cultures, Tian *et al.* (35) reported that Ang II-induced ERK phosphorylation returned to basal levels within approximately 60 min after Ang II stimulation, whereas other group reported that ERK activation remained at high levels even as long as 120 min after Ang II administration (20, 34). In other experiments using H295R cells, the ERK activation by Ang II was stimulated at the highest levels in 5 min after Ang II treatment and then gradually declined; however, it persisted even 3 (37) or 6 (38) h after Ang II exposure. In agreement with these data, Ang II effect on ERK phosphorylation was maximal at 5 min and gradually decreased; however Ang II-activated ERKs were detectable up to 4 h after Ang II exposure in our study (Fig. 6). Interestingly, longer exposure of

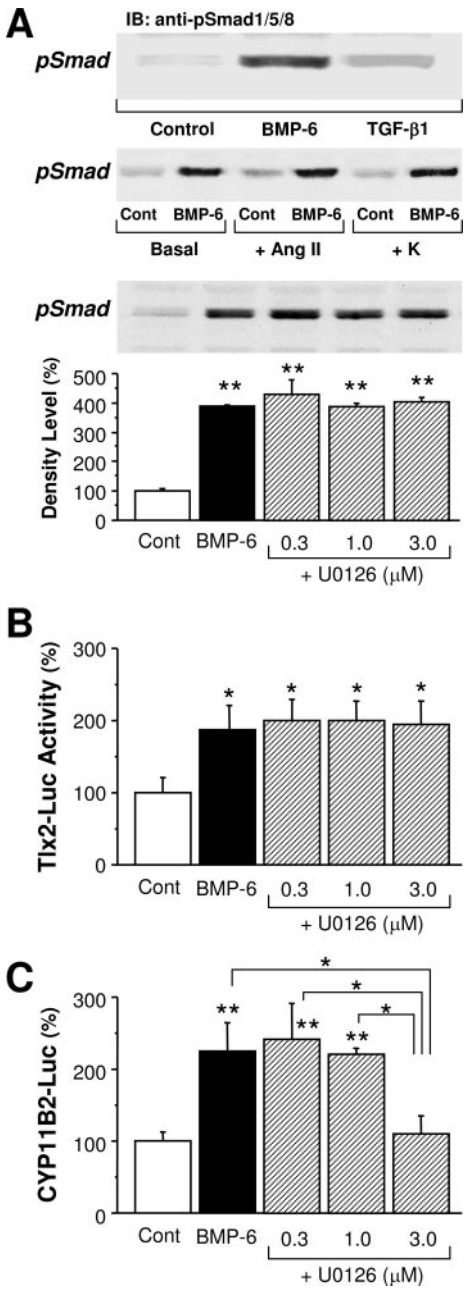

FIG. 8. Relationship between BMP and MAPK signaling in H295R cells. A, Effect of BMP-6 on Smad1/5/8 phosphorylation. Cells were precultured in fresh medium containing 0.3% NuSerum and treated with BMP-6 (100 ng/ml) or TGF-β1 (100 ng/ml) in combination with Ang II (10 nM), K (16 mM), or an ERK inhibitor, U0126 (μM). The cell lysates were subjected to immunoblotting analysis using antiphospho-Smad (pSmad) 1/5/8 antibody. The band intensity of scanned images was statistically analyzed. B and C, Transcriptional regulations of BMP signaling Tlx2 and CYP11B2 in H295R cells. Cells were transiently transfected with 1 μg each luciferase reporter plasmids of Tlx2-Luc (B) or pGL3-CYP11B2 (C) and 0.1 μg pCMV-β-gal for 24 h. The cells were then treated in fresh medium containing 0.3% NuSerum in combinations with U0126 (0.3–3.0 μM) and BMP-6 (100 ng/ml) 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. All results are shown as mean ± SEM of data from at least three separate experiments, each performed with triplicate samples. *, $P < 0.05$; **, $P < 0.01$ *vs.* control or between the indicated groups.

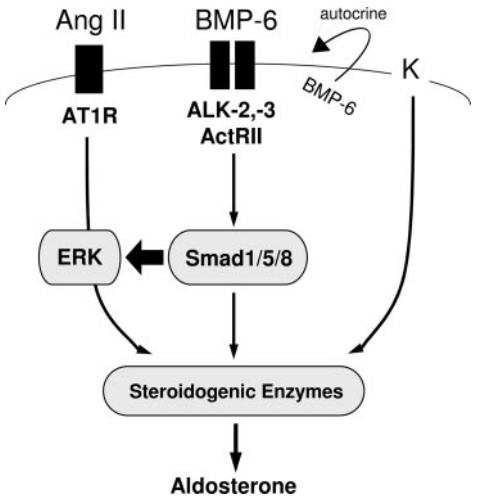

FIG. 9. A possible mechanism by which BMP-6 regulates Ang II-induced aldosterone production. Ang II stimulates aldosterone synthesis by activating ERK phosphorylation through AT1R in H295R cells although K effect on ERK activation is negligible. Importantly, BMP-6 contributes to Ang II-induced aldosterone production through activating Smad1/5/8 after the receptor binding to ALK-2 and/or ALK-3 in combination with ActRII, which sustains Ang II-induced ERK activation. A functional cross-talk between BMP-6/Smad and Ang II/ERK exists in a process of aldosterone production.

cells to Ang II in combination with BMP-6 and, in particular, the pretreatment of cells with BMP-6 significantly augmented ERK phosphorylation induced by Ang II. The effect of BMP-6 on Ang II-induced ERK activation was potently inhibited by ALK-2-ECD as well as ALK-3-ECD (Fig. 7).

Thus, the two signaling pathways of BMP-6 and ERK are functionally interrelated for aldosterone production, although the direct ERK1/2 phosphorylation due to BMP-6 was not detected by immunoblotting analysis in the present study. Given that the inhibition of ERK activation was effective for reducing BMP-6-induced CYP11B2 transcription but ineffective for inactivating BMP-6-induced Smad1/5/8 signaling, Smad activation for aldosterone stimulation can be elicited at least at the upstream of ERK activation (Fig. 9). Ang II-ERK and BMP-6-Smad pathways could be originally distinct but connected with some points of the ERK activation. However, we cannot exclude the possibility that BMP-6 may activate a common unidentified signal with K-induced pathway. Further studies for the direct evidence of protein association including Smads and ERK are necessary to confirm this provisional conclusion.

Collectively, we demonstrated the existence of functional BMP system in the human adrenocortical cell line, H295R. A new cross-talk pathway between BMP-6/Smad signaling and Ang II/ERK signaling plays key roles in the governance of aldosterone production by human adrenocortical cells (Fig. 9). Thus, BMP-6 may play critical roles in diverged aldosterone production between Ang II and K through modulating Ang II-induced ERK phosphorylation. Such an elaborate system comprised of intraadrenal BMP and the renin-Ang system may contribute to fine-tuning of aldosterone production.

Acknowledgments

We thank Dr. R. Kelly Moore for helpful discussion and critical reading of the manuscript. We also thank Drs. William E. Rainey, Jeff Wrana, and Shunichi Shimasaki for providing the plasmids of pGL3-CYP11B2, Tlx2-Luc, and pActRII/pBMPRII-DN, respectively.

Received October 3, 2005. Accepted February 24, 2006.

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This work was supported in part by Grants-in-Aid for Scientific Research (16790525), Uehara Memorial Foundation, Kanzawa Medical Research Foundation, Yamaguchi Endocrine Research Association, Life Science Foundation of Japan, and Takeda Science Foundation.

All authors have nothing to declare.

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