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Aldosterone breakthrough caused by chronic blockage of angiotensin II type 1 receptors in human adrenocortical cells: Possible involvement of bone morphogenetic protein-6 actions

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Abbreviations:
ALK, activin receptor-like kinase
ActRI and ActRII, activin type I and type II receptor
ACE, angiotensin-converting enzyme
Ang II, angiotensin II
ARB, Ang II type 1 receptor blockers
AT1R and AT2R, Ang II type 1 and type 2 receptor
BMPRI and BMPRII, BMP type I and type II receptor
CYP11B2, P450aldo gene
ECD, extracellular domain
ERK, extracellular signal-regulated kinase
MAP kinase, mitogen-activated protein kinase
P450aldo, P450 aldosterone synthase
P450scc, P450 steroid side-chain cleavage enzyme
StAR, steroidogenic acute regulatory protein
TGF-β, transforming growth factor-β

ABSTRACT

Circulating aldosterone concentrations occasionally increase after initial suppression with angiotensin II (Ang II) converting enzyme inhibitors or Ang II type 1 receptor (AT1R) blockers (ARB), a phenomenon referred to as “aldosterone breakthrough”. However, the underlying mechanism causing the aldosterone breakthrough remains unknown. Here we investigated whether aldosterone breakthrough occurs in human adrenocortical H295R cells *in vitro*. We recently reported that BMP-6, which is expressed in adrenocortical cells, enhances Ang II- but not potassium-induced aldosterone production in human adrenocortical cells. Accordingly, we examined the roles of BMP-6 in aldosterone breakthrough induced by long-term treatment with ARB. Ang II stimulated aldosterone production by adrenocortical cells. This Ang II stimulation was blocked by an ARB, candesartan. Interestingly, the candesartan effects on Ang II-induced aldosterone synthesis and CYP11B2 expression were attenuated in a course of candesartan treatment for 15 days. The impairment of candesartan effects on Ang II-induced aldosterone production was also observed in Ang II- or candesartan-pretreated cells. Levels of AT1R mRNA were not changed by chronic candesartan treatment. However, BMP-6 enhancement of Ang

II-induced ERK1/2 signaling was resistant to candesartan. The BMP-6-induced Smad1,5,8 phosphorylation and BRE-Luc activity was augmented in the presence of Ang II and candesartan in the chronic phase. Chronic Ang II exposure decreased cellular expression levels of BMP-6 and its receptors ALK-2 and ActRII mRNAs. Co-treatment with candesartan reversed the inhibitory effects of Ang II on the expression levels of these mRNAs. The breakthrough phenomenon was attenuated by neutralization of endogenous BMP-6 and ALK-2. Collectively, these data suggest that changes in BMP-6 availability and response may be involved in the occurrence of cellular escape from aldosterone suppression under chronic treatment with ARB.

INTRODUCTION

Production of aldosterone occurs in the adrenal glomerulosa, which is regulated primarily by angiotensin II (Ang II) and potassium and, to a lesser degree, adrenocorticotropin (ACTH) (1, 2). In the presence of these aldosterone stimulators, steroidogenesis in the adrenal cortex is further governed by local autocrine and/or paracrine factors (3). We previously reported the presence of a functional bone morphogenetic protein (BMP) and activin system complete with ligands including BMP-6, activins and their receptors in the human adrenocortical cell line, H295R (4). BMPs 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 (5-7), pituitary (8, 9), thyroid (10) and adrenal (4, 11) have been elucidated to date.

In human H295R adrenocortical cells, BMP-6 augments Ang II-induced aldosterone production and activin regulates ACTH-dependent aldosterone production (4). Further investigation demonstrated that BMP-6 activated Ang II-induced CYP11B2 transcription and aldosterone production; in contrast, BMP-6 had no effect on potassium-induced aldosterone production (12). Based on the established role of

the endogenous BMP system in Ang II-induced aldosterone production (12), we hypothesized that BMP-6 actions in adrenocortical cells may be involved in "aldosterone breakthrough".

Clinical trials have established that plasma aldosterone levels occasionally increase after an initial decline in some patients over the course of long-term therapy with angiotensin-converting enzyme (ACE) inhibitors and/or Ang II type 1 receptor (AT1R) blocker (ARB). Aldosterone breakthrough is a phenomenon where circulating aldosterone concentrations increase above pretreated levels after long-term therapy with ACE inhibitors (13, 14) or ARB (15). This phenomenon, termed 'aldosterone escape' or 'aldosterone breakthrough', could have important clinical consequences since increased aldosterone in a high salt state may facilitate cardiovascular and renal damages in hypertensive patients (16, 17). Involvement of various *in vivo* factors such as ACTH, electrolytes, endothelins and Ang II type 2 receptor (AT2R) actions (15, 18) have been proposed to explain this breakthrough phenomenon; however, the detailed underlying mechanism remains unknown.

In the present study, we hypothesized that local BMP-6 actions in adrenocortical cells may be involved in the mechanism of aldosterone breakthrough. To elucidate the underlying cellular mechanism by which aldosterone breakthrough

phenomenon occurs in the process of chronic AT1R blockade of human adrenocortical cells, we examined the possible role of BMP-6 in aldosterone breakthrough occurring with long-term ARB therapy in *in vitro* human adrenocortical H295R cells.

MATERIALS AND METHODS

Reagents and Supplies

A 1:1 mixture of Dulbecco's Modified Eagle's Medium/Ham's F-12 medium (DMEM/F12), penicillin-streptomycin solution, and Ang II acetate salt were purchased from Sigma-Aldrich Co. Ltd. (St. Louis, MO). Insulin-transferrin-sodium selenite plus (ITS+) was from BD Falcon (Bedford, MA). Recombinant human BMP-6, extracellular domains (ECDs) which lack transmembrane and intracellular domains of ALK-2 and ALK-6 (19), anti-human BMP-6 polyclonal antibody, and normal goat antibody were from R&D Systems (Minneapolis, MN). Candesaran, CV-11974 was provided from Takeda Chemical Industries (Osaka, Japan). Adult male spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats were purchased from Charles-River (Wilmington, MA). The animal protocols were approved by Okayama University Institutional Animal Care and Use Committee. The plasmid of BRE-Luc

containing a BMP-responsive element fused to the firefly luciferase reporter gene was kindly provided from Drs. Tetsuro Watabe and Kohei Miyazono, Tokyo University, Japan.

Cell culture and aldosterone assay

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 of potassium, 10% fetal calf serum (FCS), 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 (4, 12). To assess the effect of treatments on aldosterone secretion, monolayered cells (4×10^4 viable cells) were precultured in 96-well human fibronectin-coated plates (Biocoat®, BD-Falcon). After 48-h culture, the medium was replaced with fresh medium containing 4 mM of potassium and 1% FCS with or without various combinations of Ang II, candesartan, BMP-6, ALK-2- or ALK-6-ECDs, and anti-BMP-6 or control IgG at indicated concentrations. H295R cells were then cultured for indicated periods and the accumulated levels of aldosterone in the conditioned media were determined by radioimmunoassay (SPAC-S aldosterone, TFB Co., Tokyo, Japan).

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

H295R cells (1 × 10^6 viable cells) were grown in 6-well plates and the medium was replaced with fresh DMEM/F12 containing 4 mM of potassium and 1% FCS. The cells were treated with Ang II, aldosterone, or combinations of the reagents including candesartan and BMP-6 as indicated concentrations. After culture for indicated periods, the medium was removed and total cellular RNA was extracted by isothiocyanate-acidphenol-chloroform methods using TRIzol® (Invitrogen Corp., Carlsbad, CA) and quantified by measuring absorbance at 260 nm and stored at -80°C until assay. Oligonucleotides used for RT-PCR were custom-ordered from Invitrogen Corp. 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 First-Strand cDNA Synthesis System® (Invitrogen Corp.) with random hexamer (2 ng/μl), reverse transcriptase (200 U) and deoxynucleotide triphosphate (dNTP; 0.5 mM). The primer settings for StAR, P450scc, CYP11B2, BMP-6 (4, 12, 20), ALK-2, ALK-3, ActRII, Smad1, Smad4, Smad5, and a house-keeping gene, ribosomal protein L19 (RPL19) (8, 21), were followed by our earlier reports. For the quantification, real-time

PCR was performed using 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 manufacture's protocol. 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 RPL19 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 (~60% confluency) were transiently transfected with 500 ng of each luciferase reporter plasmid (BRE-Luc) and 0.1 μg of cytomegalovirus-β-galactosidase plasmid (pCMV-β-gal) using FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN) after 1-, 7- and 15-day pretreatment with a combination of Ang II and <u>candesartan</u>. The cells were then treated with BMP-6 at indicated concentrations <u>in DMEM/F12 containing 4 mM of potassium and 1% FCS for 24 h.</u> 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 as we previously reported (22). The

data were shown as the ratio of luciferase to β-gal activity.

Western immunoblot analysis

Cells (1 × 10^6 viable cells) were seeded in 12-well human fibronectin-coated plates (Biocoat®, BD-Falcon) in DMEM/F12. After 24-h preculture with serum-free conditions containing 4 mM of potassium, indicated concentrations of BMP-6, Ang II and candesartan were added to the culture media. Following stimulation by hormones and growth factors in the indicated time course, cells were solubilized in 100 μl RIPA 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 (10). The cell lysates were then subjected to SDS-PAGE immunoblotting analysis using anti-phospho-, anti-total-extracellular signal-regulated kinase (ERK) 1/2 MAP kinases antibodies and anti-phospho-Smad1/5/8 antibody (Cell signaling Technology, Inc., Beverly, MA).

Immunofluorescence microscopy

For immunofluorescence study, H295R cells were precultured in serum-free DMEM/F12 containing 4 mM of potassium using chamber slides (Nalge Nunc Int.,

Naperville, IL). Cells at ~50% confluency were pretreated with a combination of Ang II and candesartan for 1 h and then treated with BMP-6 (100 ng/ml) for 1 h. Cells were then 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.) for 1 h and washed three times with PBS. 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 fluorescent microscope.

Immunohistochemistry study

For the immunohistochemical analyses, 4 μm-thick sections of formalin-fixed and paraffin-embedded adrenal tissues were dewaxed and rehydrated, and antigen retrieval was performed by heating for 15 min in a 10 mM citrate buffer at pH 6.0. The sections were reacted with gout polyclonal antibody for BMP-6 (R&D Systems) diluted at 10 μg/ml for 16 h at 4°C, and then were subsequently stained using the universal immuno-peroxidase polymer method with Histofine staining kit (Nichirei Corp., Tokyo, Japan) according to the manufacturer's protocol. Positive reaction was visualized with 3,3'-diaminobenzidine tetrahydrochloride, followed by counterstaining with

hematoxylin. For negative controls, normal goat antibody was used instead of primary antibodies, and no specific immunoreactivity was detected in these sections.

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 (PLSD) test (StatView 5.0 software, Abacus Concepts, Inc., Berkeley, CA). P values < 0.05 were accepted as statistically significant.

RESULTS

The *in vitro* effects of an ARB, candesartan on Ang II-induced aldosterone production were examined using human adrenocortical H295R cells. Aldosterone levels were measured in the medium accumulated during the indicated culture conditions. As shown in **Fig. 1A**, Ang II (1 to 30 nM) stimulated aldosterone production for 24-h culture. Ang II-induced aldosterone production for 24 h was blocked by candesartan (0.3 to 10 nM) in a dose-dependent manner. Based on this

result, 1.0 nM candesartan was selected as the IC_{50} value for Ang II (10 nM)-induced aldosoterone levels in order to examine long-term effects of candesartan on Ang II-induced aldosterone accumulation in the medium by H295R cells. The time-course reduction of Ang II-induced aldosterone accumulation by candesartan treatment was demonstrated in **Fig. 1B**. Accumulated aldosterone concentration attained 3 to 4 nM in our culture conditions for 15 days. Candesartan treatment inhibited Ang II-induced aldosterone levels by 50% to 60%, an effect that was sustained for up to 10 days in culture conditions. After 13- to 15-day treatment with candesartan, the levels of aldosterone synthesis rebounded to 80% to 85% of Ang II-induced aldosterone levels (**Fig. 1B**).

To further investigate the dynamics of the impairment of aldosterone suppression by long-term candesartan action, 24-h production of aldosterone was evaluated in the cells pretreated with Ang II and candesartan for 3 days. During the culture period, culture medium was completely changed into fresh media containing a steady concentration (4 mM) of potassium for each 24 h. In non-pretreated conditions (**Fig. 2A**), aldosterone production at 24 h was reduced by candesartan treatment to 25% of the Ang II-induced levels. This candesartan suppression was impaired after 13 days in culture; and aldosterone production was nearly restored to the levels of day 1 of

candesartan treatment by the 15th day of culture (**Fig. 2A**). The phenomenon of impaired candesartan suppression was also detected in the cells pretreated with candesartan (**Fig. 2B**) as well as Ang II (**Fig. 2C**). During the observation period, maximal suppression of aldosterone levels was seen between 7- to 9-day culture with candesartan. The breakthrough phenomenon from candesartan effects on Ang II-induced aldosterone production was detected after 15-day culture conditions regardless of the pretreatment with Ang II and candesartan.

To characterize the changes of Ang II sensitivity and the expression of steroidogenic enzymes in candesartan-treated H295R cells, levels of AT1R, StAR, P450scc and CYP11B2 mRNA were examined by real-time PCR analysis (**Fig. 3**). The expression levels of AT1R were not significantly changed by chronic candesartan treatment during 15-day culture, suggesting that downregulation of AT1R was not critical in the aldosterone breakthrough phenomenon. After 1-day culture with Ang II, mRNA levels of StAR, P450scc and CYP11B2 were significantly increased. Co-treatment with candesartan for 1 day restored the Ang II-induced expression of steroidogenic factors including StAR and P450scc to the basal levels, while the CYP11B2 level was partially reversed by candesartan for 1 day (**Fig. 3**). At the 7th day of culture, Ang II significantly induced StAR and CYP11B2 expression, which was

restored by candesartan treatment. After 15 days of culture, Ang II induction of StAR and P450scc expression became insignificant, while Ang II augmentation of CYP11B2 expression was persistent throughout the 15-day culture (**Fig. 3**). Of note, candesartan treatment exerted significant suppression of Ang II-induced CYP11B2 mRNA levels on 1- and 7-day culture; however, the inhibitory effects by candesartan were abolished by the 15th day of culture (**Fig. 3**). Thus, the candesartan effects on reducing Ang II-induced CYP11B2 expression and Ang II-induced aldosterone synthesis were reversed during the course of candesartan treatment over a 15-day culture, *i.e.,* a possible cellular aldosterone breakthrough was demonstrated *in vitro*.

Based on our earlier study (12), BMP-6 enhances Ang II-induced aldosterone production through ERK1/2 signaling. To investigate the involvement of BMP-6 in reducing aldosterone suppression therefore, aldosterone breakthrough by candesartan, we assessed the changes of ERK1/2 activation induced by Ang II in the presence and absence of candesartan and BMP-6. As shown in **Fig. 4**, the stimulating effects of Ang II on ERK1/2 phosphorylation were blocked by candesartan treatment for 1-, 7- and 15-day culture. In the presence of BMP-6, the Ang II-induced ERK1/2 phosphorylation was enhanced and candesartan treatment was ineffective in suppressing Ang II-induced ERK1/2 phosphorylation for 1-, 7- and 15-day culture (**Fig. 4**). These

results suggest that BMP-6 enhancement of Ang II-induced ERK1/2 activation is possibly involved in the mechanism of aldosterone breakthrough from chronic candesartan treatment in adrenocortical cells.

We next examined changes of BMP-6 signaling in adrenocortical cells in the presence of Ang II and candesartan. BMP-6-induced activation of Smad1,5,8 phoshphorylation was detected by immunofluorescence (**Fig. 5A**) and Western immunoblot (**Fig. 5B**). Nuclear localization of phospho-Smad1,5,8 proteins was detected in cells stimulated by BMP-6 for 1 h, and pretreatment with Ang II and candesartan for 24 h had no effect on the nuclear localization (**Fig. 5A**). To investigate changes in the intensity of phospho-Smad1,5,8 signaling during 15-day culture, H295R pretreated with Ang II and candesartan for 1, 7 and 15 days were stimulated by BMP-6, and then cell lysates were used for immunoblotting analysis for phospho-Smad1,5,8 detection after adjustment of total protein contents (**Fig. 5B**). On 1-day culture, there were no differences in BMP-6 responsiveness between cells treated with Ang II and Ang II / candesartan cotreatment. However, after 7 days of culture Smad1,5,8 activation was impaired in Ang II-treated cells as compared to cells co-treated with Ang II / candesartan. On 15-day culture, Ang II-treated cells had a weak response to exogenous BMP-6 stimulation compared with those treated with Ang II plus

candesartan (**Fig. 5B**). To quantify BMP-6 signaling in H295R cells, a BMP-responsive reporter construct, BRE-luc, was utilized (**Fig. 6**). BMP-6-induced BRE-luc activity was reduced in cells treated with Ang II for 15 days although it was not significantly changed by Ang II treatment for 1- and 7-day culture. However, this reduction was not observed in cells co-treated with candesartan for 15 days (**Fig. 6**). Taken together with the results in **Fig. 5B**, it is likely that chronic Ang II treatment reduces BMP-6 signaling, whereas the co-treatment with candesartan abolishes Ang II-induced reduction of BMP-6-to-Smad1,5,8 signaling activity in adrenocortical cells.

To examine the mechanism by which Ang II and candesartan treatment affects BMP-6 signaling pathway in H295R cells, the expression of key molecules for BMP-6 actions in H295R cells were evaluated by quantitative PCR. As shown in **Fig. 7A**, BMP-6 ligand expression appeared to be reduced in the course of 15-day Ang II treatment. The reduction of BMP-6 was blocked by the addition of candesartan. The expression of functional BMP-6 receptors, ActRII and ALK-2, was also reduced in cells treated with Ang II for 15 days, an effect that was attenuated in cells co-treated with candesartan (**Fig. 7A**). ALK-3 expression was not affected by Ang II as well as candesartan treatment in H295R cells. We also examined the Smad mRNA expression levels. Based on our earlier data by cDNA array analysis (20), the predominant Smad

molecules expressed in H295R cells are Smad1, 4 and 5. As shown in **Fig. 7B**, Ang II treatment had a moderate effect on reducing Smad1 and Smad5, but not Smad4, mRNA levels. Unlike the results observed with respect to ActRII and ALK-2, co-treatment with candesartan had no significant effects on the expression level of Smad1,4 and 5 in cells treated with Ang II for 1, 7 or 15 days (**Fig. 7B**). In addition, we examined effects of high concentrations of aldosterone (10 to 100 nM) on the expression levels of BMP-6 and its receptors ALK-2 and ActRII. As shown in **Fig, 7C**, treatment with a high concentration of aldosterone (100 nM) transiently decreased BMP-6 and ActRII mRNA levels; however, the changes were restored by the time when aldosterone breakthrough occurs in H295R cells.

To elucidate the functional roles of the BMP-6 system in the aldosterone breakthrough phenomenon, we attempted to ablate the endogenous BMP-6 signaling with a neutralizing antibody against BMP-6 ligand or extracellular domains (ECDs) of BMP type I receptors that harmlessly block BMP-6 binding to endogenous ALK-2 (12, 19). ALK-6-ECD was used as a negative control since ALK-6 was not expressed in H295R cells (12). As shown in **Fig. 8A**, the escape phenomenon by candesartan effects was ameliorated on cultures for 13 days in the presence of ALK-2-ECD, whereas ALK-6-ECD had no significant effects on the breakthrough phenomenon. In

addition, the neutralization of BMP-6 by BMP-6-IgG also attenuated the breakthrough phenomenon in contrast to the cells treated with control IgG (**Fig. 8B**). Thus, endogenous BMP-6/ALK-2 system is possibly involved in the mechanism by which chronic candesartan treatment attenuates the suppression of Ang II-induced aldosterone production.

As shown in **Fig. 9**, immunohistochemical staining demonstrated that BMP-6 protein was broadly expressed in the adrenal cortex of 12-week-old male Wistar-Kyoto (WKY) rats. Notably, BMP-6 expression was detected most strongly in the zona glomerulosa of adrenal cortex obtained from age-matched male spontaneously hypertensive rats (SHR). This finding infers *in vivo* roles of BMP-6 in regulating aldosterone production as an autocrine/paracrine factor in the adrenal glomerulosa.

DISCUSSION

Aldosterone breakthrough is a state of sustained aldosterone synthesis in the adrenal during relative long-term treatment with ACE inhibitors (13, 14) and/or ARB (15, 23). Based on literature reports (24), the incidence of the aldosterone breakthrough phenomenon ranges from 10% over 6 months to 53% over 1 year. These

figures, however, depend on the definition of "aldosterone breakthrough" in the respective studies. For example, in studies that defined breakthrough as any increase from individual basal levels of pre-ACEI and/or ARB treatment, the incidence is unexpectedly high ranging from 40 to 53% (24). No specific association between variety or dose of ACE inhibitor and/or ARB and incidence of aldosterone breakthrough has been reported (25).

Extraadrenal aldosterone production in the heart and blood vessels (26, 27) may also contribute to aldosterone breakthrough phenomenon. Nevertheless, there is still an argument against the pathophysiological significance of local aldosterone because of its very low production (28). It is therefore important to understand how the regulation of aldosterone synthesis in the adrenal cortex may be involved as a causative mechanism in aldosterone breakthrough. We studied the mechanism of aldosterone breakthrough phenomenon in cultured adrenocortical cells. Aldosterone breakthrough is clinically considered as an elevation of plasma aldosterone concentration after treatment with ACEI or ARB as compared to pretreatment values. In the present *in vitro* study, the significant impairment of ARB suppression on Ang II-induced aldosterone production was defined as the cellular breakthrough phenomenon.

We recently reported that BMP-6, which is expressed in adrenal cortex, enhances Ang II- but not potassium-induced aldosterone production in human adrenocortical H295R cells (12). Molecular approaches have shown that BMP-6 augments Ang II-induced aldosterone production by upregulating ERK1/2 signaling via receptor complexes composed of type I receptors including ALK-2 and/or ALK-3, and ActRII (12). In the present study, reduction of ARB effects on Ang II-induced aldosterone production was also shown in adrenocortical cells *in vitro*. Expression levels of AT1R mRNA were not directly involved in this phenomenon. Notably, the enhancing effects of BMP-6 on Ang II-induced ERK1/2 signaling were resistant to ARB. BMP-6-induced Smad1,5,8 activation was in turn amplified in cells chronically treated with Ang II plus an ARB, candesartan, compared with cells treated with Ang II alone.

We have earlier reported that cellular BMP-6 mRNA expression was transiently reduced at 6 to 12 h after stimulation with Ang II and potassium in H295R cells, while it was not affected by treatments with ACTH and forskolin (20). Since BMP-6 plays critical roles in the differential mechanisms regulating aldosterone production by Ang II and potassium through modulating Ang II-induced MAP kinase pathway (12), this autocrine regulation most likely contributes to fine-tuning

aldosterone production in the adrenal cortex. In the present study we found that the expression of BMP-6 mRNA and its receptor mRNAs, including ALK-2 and ActRII, were decreased by long-term Ang II exposure but restored by co-treatment with ARB. In contrast, long-term exposure of aldosterone at high concentrations had no significant effects on the expression of BMP-6, ALK-2 and ActRII in adrenocortical cells. Importantly, neutralization of ALK-2 and BMP-6 function attenuated the escaping effect from aldosterone suppression by ARB. Given that inhibition of endogenous BMP-6 by neutralizing antibodies selectively reduced Ang II- but not potassium-induced aldosterone synthesis (12), it is likely that endogenous BMP-6 plays an important autocrine role in regulating aldosterone production caused by Ang II. Changes in the bioavailability of BMP-6 and cellular BMP responsiveness may be, at least in part, involved in the occurrence of cellular escape from aldosterone suppression under chronic treatment with ARB (**Fig. 10**).

Taken together, the presence of the breakthrough mechanisms cannot be explained solely by the effect of Ang II through the AT1R. Factors that regulate aldosterone production other than Ang II and potassium have been suggested, which either stimulate (*e.g.* corticotrophin, prolactin, serotonin, vasopressin, endothelins) or inhibit (*e.g.* atrial natriuretic hormone; ANP, nitric oxide, somatostatin) circulating

aldosterone levels. Among these factors, *in vivo* physiological significance has been only demonstrated for ACTH and ANP (29). Since no significant changes in plasma cortisol concentrations during ACE inhibition are observed (30), it is likely that ACTH has little involvement in aldosterone escape during ACE inhibitor or ARB therapy. Potassium levels rather, than the renin-angiotensin system, stimulate aldosterone secretion during salt restriction (31). However, no significant correlation between plasma aldosterone concentration and serum potassium concentration were shown in patients treated with long-term ACE inhibitor therapy (32). Nevertheless, small changes in serum potassium concentration within the physiological range may affect aldosterone secretion.

A possible role for Ang II action through AT2R is worthy of consideration to explain why ARB may not be effective in lowering plasma aldosterone concentration in all cases. In this regard, Naruse *et al.* (15) reported that aldosterone breakthrough occurred during long-term ARB candesartan therapy in stroke-prone spontaneously hypertensive rats, and when they administered an AT2R antagonist concomitantly with candesartan, plasma aldosterone concentrations decreased significantly. Ang II action in aldosterone synthesis is mediated solely by AT1R in H295R cells since AT2R is not expressed (33). Regarding endothelins, the plasma endothelin-1 (ET-1) concentration

tends to be elevated in patients with chronic heart failure (34). Since ET-1 stimulates aldosterone secretion and adrenocortical growth, it may also be a possible candidate involved in the mechanism of aldosterone breakthrough (35).

Collectively, the existence of cellular aldosterone breakthrough was demonstrated in the current study. In a clinical aspect, aldosterone breakthrough occurs in a significant proportion of patients on long-term ACE inhibitor and/or ARB therapy, which seems likely to be originated in the adrenocortical cells *per se*. The renin-angiotensin-aldosterone (RAA) system is a key regulator of blood pressure and fluid homeostasis. The main effector molecule of this system, angiotensin II, is produced from the substrate angiotensinogen through sequential enzymatic cleavages by renin and ACE. In the circulating system, the amount of renin in the plasma is a key rate-limiting step determining the overall RAA system activity. Since the activity of the circulating RAA system is precisely calibrated by signals from the kidney linked to blood pressure and intake of salt and water, the counter-regulatory stimuli to RAA system including elevated potassium levels, use of diuretics, decompensated heart failure, and declines in blood pressure may also be involved in the mechanism of aldosterone breakthrough.

Renin inhibitors suppress the RAA system in its earliest stage without accumulation of active precursor substances. This new class of antihypertensive medication may, therefore, be associated with less aldosterone breakthrough than conventional RAA system blockade with ACEI and/or ARB (36). The breakthrough phenomenon might be associated with important cardiovascular and renal outcomes, including left ventricular hypertrophy, poor exercise tolerance, refractory proteinuria, and declining glomerular filtration rate (24). It is therefore important to characterize molecular cause of aldosterone breakthrough. If aldosterone breakthrough is detected in the setting of stable potassium and salt balance, then the addition of aldosterone antagonists or renin inhibitors to conventional heart and kidney failure regimens could improve clinical outcomes.

Based on the present *in vitro* study, changes in the bioavailability of BMP-6 and cellular BMP responsiveness are, at least in part, involved in the occurrence of cellular escape from aldosterone suppression under chronic AT1 blockade. Further approaches would be necessary to determine the factors that control BMP-6 expression in the adrenal and the pathophysiological roles of adrenocoritcal BMP-6 system *in vitro* in studying aldosterone breakthrough.

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REFERENCES

1. **Foster RH, MacFarlane CH, Bustamante MO** 1997 Recent progress in understanding aldosterone secretion. Gen Pharmacol 28:647-651
2. **Lumbers ER** 1999 Angiotensin and aldosterone. Regul Pept 80:91-100
3. **Ehrhart-Bornstein M, Hinson JP, Bornstein SR, Scherbaum WA, Vinson GP** 1998 Intraadrenal interactions in the regulation of adrenocortical steroidogenesis. Endocr Rev 19:101-143
4. **Suzuki J, Otsuka F, Inagaki K, Takeda M, Ogura T, Makino H** 2004 Novel action of activin and bone morphogenetic protein in regulating aldosterone production by human adrenocortical cells. Endocrinology 145:639-649
5. **Otsuka F, Yao Z, Lee TH, Yamamoto S, Erickson GF, Shimasaki S** 2000 Bone morphogenetic protein-15: Identification of target cells and biological functions. J Biol Chem 275:39523-39528
6. **Shimasaki S, Moore RK, Otsuka F, Erickson GF** 2004 The bone morphogenetic protein system in mammalian reproduction. Endocr Rev 25:72-101
7. **Otsuka F, Moore RK, Shimasaki S** 2001 Biological function and cellular mechanism of bone morphogenetic protein-6 in the ovary. J Biol Chem 276:32889-32895
8. **Takeda M, Otsuka F, Suzuki J, Kishida M, Ogura T, Tamiya T, Makino H** 2003 Involvement of activin/BMP system in development of human pituitary gonadotropinomas and nonfunctioning adenomas. Biochem Biophys Res Commun 306:812-818
9. **Otsuka F, Shimasaki S** 2002 A novel function of bone morphogenetic protein-15 in the pituitary: selective synthesis and secretion of FSH by gonadotropes. Endocrinology 143:4938-4941
10. **Suzuki J, Otsuka F, Takeda M, Inagaki K, Miyoshi T, Mimura Y, Ogura T, Doihara H, Makino H** 2005 Functional roles of the bone morphogenetic protein system in thyrotropin signaling in porcine thyroid cells. Biochem Biophys Res Commun 327:1124-1130
11. **Kano Y, Otsuka F, Takeda M, Suzuki J, Inagaki K, Miyoshi T, Miyamoto M, Otani H, Ogura T, Makino H** 2005 Regulatory roles of bone morphogenetic proteins and glucocorticoids in catecholamine production by rat

pheochromocytoma cells. Endocrinology 146:5332-5340

12. **Inagaki K, Otsuka F, Suzuki J, Kano Y, Takeda M, Miyoshi T, Otani H, Mimura Y, Ogura T, Makino H** 2006 Involvement of bone morphogenetic protein-6 in differential regulation of aldosterone production by angiotensin II and potassium in human adrenocortical cells. Endocrinology 147:2681-2689
13. **Staessen J, Lijnen P, Fagard R, Verschueren LJ, Amery A** 1981 Rise in plasma concentration of aldosterone during long-term angiotensin II suppression. J Endocrinol 91:457-465
14. **Lijnen P, Staessen J, Fagard R, Amery A** 1982 Increase in plasma aldosterone during prolonged captopril treatment. Am J Cardiol 49:1561-1563
15. **Naruse M, Tanabe A, Sato A, Takagi S, Tsuchiya K, Imaki T, Takano K** 2002 Aldosterone breakthrough during angiotensin II receptor antagonist therapy in stroke-prone spontaneously hypertensive rats. Hypertension 40:28-33
16. **Sato A, Saruta T** 2001 Aldosterone escape during angiotensin-converting enzyme inhibitor therapy in essential hypertensive patients with left ventricular hypertrophy. J Int Med Res 29:13-21
17. **Sato A, Hayashi K, Naruse M, Saruta T** 2003 Effectiveness of aldosterone blockade in patients with diabetic nephropathy. Hypertension 41:64-68
18. **Sato A, Saruta T** 2003 Aldosterone breakthrough during angiotensin-converting enzyme inhibitor therapy. Am J Hypertens 16:781-788
19. **Moore RK, Otsuka F, Shimasaki S** 2003 Molecular basis of bone morphogenetic protein-15 signaling in granulosa cells. J Biol Chem 278:304-310
20. **Inagaki K, Otsuka F, Suzuki J, Otani H, Takeda M, Kano Y, Miyoshi T, Yamashita M, Ogura T, Makino H** 2007 Regulatory expression of bone morphogenetic protein-6 system in aldosterone production by human adrenocortical cells. Regul Pept 138:133-140
21. **Miyoshi T, Otsuka F, Suzuki J, Takeda M, Inagaki K, Kano Y, Otani H, Mimura Y, Ogura T, Makino H** 2006 Mutual regulation of follicle-stimulating hormone signaling and bone morphogenetic protein system in human granulosa cells. Biol Reprod 74:1073-1082
22. **Otani H, Otsuka F, Inagaki K, Takeda M, Miyoshi T, Suzuki J, Mukai T, Ogura T, Makino H** 2007 Antagonistic effects of bone morphogenetic protein-4 and -7 on renal mesangial cell proliferation induced by aldosterone through MAPK activation. Am J Physiol Renal Physiol 292:F1513-1525
23. **Horita Y, Taura K, Taguchi T, Furusu A, Kohno S** 2006 Aldosterone

breakthrough during therapy with angiotensin-converting enzyme inhibitors and angiotensin II receptor blockers in proteinuric patients with immunoglobulin A nephropathy. Nephrology (Carlton) 11:462-466

24. **Bomback AS, Klemmer PJ** 2007 The incidence and implications of aldosterone breakthrough. Nat Clin Pract Nephrol 3:486-492
25. **MacFadyen RJ, Lee AF, Morton JJ, Pringle SD, Struthers AD** 1999 How often are angiotensin II and aldosterone concentrations raised during chronic ACE inhibitor treatment in cardiac failure? Heart 82:57-61
26. **Mizuno Y, Yoshimura M, Yasue H, Sakamoto T, Ogawa H, Kugiyama K, Harada E, Nakayama M, Nakamura S, Ito T, Shimasaki Y, Saito Y, Nakao K** 2001 Aldosterone production is activated in failing ventricle in humans. Circulation 103:72-77
27. **Hatakeyama H, Miyamori I, Fujita T, Takeda Y, Takeda R, Yamamoto H** 1994 Vascular aldosterone. Biosynthesis and a link to angiotensin II-induced hypertrophy of vascular smooth muscle cells. J Biol Chem 269:24316-24320
28. **Ahmad N, Romero DG, Gomez-Sanchez EP, Gomez-Sanchez CE** 2004 Do human vascular endothelial cells produce aldosterone? Endocrinology 145:3626-3629
29. **Spat A, Hunyady L** 2004 Control of aldosterone secretion: a model for convergence in cellular signaling pathways. Physiol Rev 84:489-539
30. **Johns DW, Baker KM, Ayers CR, Vaughan ED, Jr., Carey RM, Peach MJ, Yancey MR, Ortt EM, Williams SC** 1980 Acute and chronic effect of captropril in hypertensive patients. Hypertension 2:567-575
31. **Okubo S, Niimura F, Nishimura H, Takemoto F, Fogo A, Matsusaka T, Ichikawa I** 1997 Angiotensin-independent mechanism for aldosterone synthesis during chronic extracellular fluid volume depletion. J Clin Invest 99:855-860
32. **Jorde UP, Vittorio T, Katz SD, Colombo PC, Latif F, Le Jemtel TH** 2002 Elevated plasma aldosterone levels despite complete inhibition of the vascular angiotensin-converting enzyme in chronic heart failure. Circulation 106:1055-1057
33. **Bird IM, Mason JI, Rainey WE** 1994 Regulation of type 1 angiotensin II receptor messenger ribonucleic acid expression in human adrenocortical carcinoma H295 cells. Endocrinology 134:2468-2474
34. **McMurray JJ, Ray SG, Abdullah I, Dargie HJ, Morton JJ** 1992 Plasma endothelin in chronic heart failure. Circulation 85:1374-1379
35. **Rossi GP** 2006 Aldosterone breakthrough during RAS blockade: a role for

endothelins and their antagonists? Curr Hypertens Rep 8:262-268

36. **Crowley SD, Gurley SB, Oliverio MI, Pazmino AK, Griffiths R, Flannery PJ, Spurney RF, Kim HS, Smithies O, Le TH, Coffman TM** 2005 Distinct roles for the kidney and systemic tissues in blood pressure regulation by the renin-angiotensin system. J Clin Invest 115:1092-1099

FIGURE LEGENDS

Fig. 1. Effects of ARB treatment on Ang II-induced aldosterone production by H295R cells. A) Dose-dependent suppression of aldosterone (Aldo) by 24-h treatment with Ang II and an ARB, candesartan (CV). After preculture in 96-well plates (4×10^4 viable cells), culture medium was replaced <u>with fresh DMEM/F12 containing 4 mM of potassium and low (1%) concentration of FCS.</u> Cells were then treated with indicated concentrations of Ang II (nM) in the absence or presence of CV (0.3 to 10 nM) for 24 h. Aldo levels in the conditioned medium were determined by radioimmunoassay. Results are shown as mean ± SEM of data from each performed with triplicate samples. *, $P < 0.05$ and **, $P < 0.01$ vs. the group of CV = 0 nM at each Ang II concentration; #, $P < 0.01$ vs. group of Ang II = 0 nM. B) Long-term effects of CV on Ang II-induced Aldo accumulation. Cells were treated with Ang II (10 nM) in the absence or presence of CV (IC_{50} = 1.0 nM) and Aldo levels were determined in the conditioned medium accumulated for 1 to 15 days. The percent CV suppression of Ang II-induced Aldo levels was shown during the culture. Results are shown as mean ± SEM of data from at least three separate experiments, each performed with triplicate samples. Bars with different letters indicate that group means are significantly different at $P < 0.05$.

Fig. 2. Long-term effects of ARB on Ang II-induced aldosterone secretion. After preculture in 96-well plates (4×10^4 viable cells) cells were pretreated with an ARB, candesartan (CV; 1.0 nM; B) or Ang II (10 nM; C) for 3 days. To determine 24-h production of aldosterone (Aldo), culture medium was completely replaced with fresh DMEM/F12 containing 4 mM of potassium, 1% FCS and Ang II (10 nM) in the absence or presence of CV (1.0 nM) every 24 h, and then Aldo levels for 24-h culture medium was determined by radioimmunoassay. Results are shown as mean ± SEM of data from at least three separate experiments, each performed with triplicate samples. *, $P < 0.05$ and **, $P < 0.01$ vs. Day 1 values of Ang II + CV groups or between the indicated groups.

Fig. 3. Effects of Ang II and ARB treatment on AT1R, StAR, P450scc and CYP11B2 expression in H295R cells. After preculture, cells were treated with Ang II (10 nM) in the absence or presence of an ARB, candesartan (CV; 1.0 nM) for 1, 7 and 15 days in DMEM/F12 containing 4 mM of potassium and 1% FCS. Total cellular RNA was extracted and subjected to RT reaction. For the quantification of AT1R, StAR, P450scc and CYP11B2 mRNA levels, real-time PCR analysis was performed.

The expression levels of target genes were standardized by RPL19 level in each sample. Results are shown as mean ± SEM of data from at least three separate experiments, each performed with triplicate samples. *, $P < 0.05$ and **, $P < 0.01$ as compared to each control group of Day 1, 7 and 15.

Fig. 4. Effects of ARB on ERK1/2 phosphorylation induced by Ang II and BMP-6. After preculture, cells were pretreated with or without an ARB, candesartan (CV; 1.0 nM) for 1, 7 and 15 days, and then cells were stimulated with Ang II (10 nM) and BMP-6 (100 ng/ml) in serum-free DMEM/F12 containing 4 mM of potassium. After the stimulation for 60 min, cells were lysed and subjected to SDS-PAGE and immunoblotting analysis using anti-pSmad1,5,8, anti-pERK1/2 and anti-tERK1/2 antibodies. Results shown are representative of those obtained from three independent experiments.

Fig. 5. Effects of Ang II and ARB on Smad1,5,8 phosphorylation in H295R cells. A) Cells were treated with Ang II (10 nM) and an ARB, candesartan (CV; 1.0 nM) for 1 h and the cells were stimulated with BMP-6 (100 ng/ml) for 1 h in serum-free DMEM/F12 containing 4 mM of potassium. Immunofluorescence studies were

performed with pSmad1,5,8 antibody. B) Cells were pretreated with Ang II (10 nM) and CV (1.0 nM) for 1 to 15 days and then stimulated with BMP-6 (100 ng/ml) for 1 h and 3 h in serum-free DMEM/F12 containing 4 mM of potassium. Total cell lysates were subjected to evaluate Smad1,5,8 phosphorylation by Western immunoblotting analysis. Results shown are representative of those obtained from three independent experiments.

Fig. 6. Long-term effects of Ang II and ARB on BMP-6 signaling in H295R cells. Cells were pretreated with Ang II (10 nM) and an ARB, candesartan (CV; 1.0 nM) for 1 to 15 days, and then transiently transfected with a BMP-responsive reporter construct, BRE-Luc plasmid (500 ng) and pCMV-β-gal. The cells were then treated with BMP-6 (100 ng/ml) for 24 h in DMEM/F12 containing 4 mM of potassium and 1% FCS. The cells were washed with PBS, lysed and the luciferase activity and β-galactosidase (β-gal) activity were measured by luminometer. The data were analyzed as the ratio of luciferase to β-gal activity. Results are shown as mean ± SEM of data from at least three separate experiments, each performed with triplicate samples. *, $P < 0.05$ and **, $P < 0.01$ vs. each control group or between the indicated groups.

Fig. 7. Effects of Ang II, ARB and aldosterone on BMP-6, BMP-6 receptor and Smads expression. A), B) After preculture, cells were treated with Ang II (10 nM) in the absence or presence of an ARB, candesartan (CV; 1.0 nM) for 1, 7 and 15 days in DMEM/F12 containing 4 mM of potassium and 1% FCS. Total cellular RNA was extracted and subjected to RT reaction. For the quantification of A) BMP-6, ALK-2, ALK-3, ActRII, and B) Smad1, 4 and 5 mRNA levels, real-time PCR analysis was performed. C) Cells were treated with aldosteone (Aldo; 10 and 100 nM) for 1, 7 and 15 days in DMEM/F12 containing 4 mM of potassium and 1% FCS, and then total cellular RNA was subjected to RT reaction and real-time PCR for the quantification of BMP-6, ALK-2 and ActRII. The expression levels of target genes were standardized by RPL19 level in each sample. Results are shown as mean ± SEM of data from at least three separate experiments, each performed with triplicate samples. *, $P < 0.05$ and **, $P < 0.01$ vs. each control group or between the indicated groups.

Fig. 8. Effect of neutralization of ALK-2 and BMP-6 on the aldosterone breakthrough phenomenon. After preculture in 96-well plates (4×10^4 viable cells) with fresh DMEM/F12 containing 4 mM of potassium and 1% FCS, cells were treated with Ang II (10 nM) and an ARB, candesartan (CV; 1.0 nM) for 7 to 13 days. Cells

were then treated with A) extracellular domains (ECDs; 1.0 μg/ml) that lack transmembrane and intracellular domains of ALK-2 or ALK-6; and B) anti-BMP-6 neutralizing IgG (1.0 μg/ml) or control IgG (1.0 μg/ml) for 24 h before each collection of the conditioned medium. Results are shown as mean ± SEM of data from at least three separate experiments, each performed with triplicate samples. Bars with 'a' and 'b' indicate significant difference from each control (Ang II group) level at $P < 0.05$; and the bars with different letters indicate that group means are significantly different at $P < 0.05$.

Fig. 9. Expression of BMP-6 protein in rat adrenal cortex. A) Control study for immunohistochemistry in the adrenal cortex sections derived from 12-week-old male Wistar-Kyoto (WKY) rats. Normal goat antibody (control antibody) was used for primary antibody instead of anti-BMP-6 antibody. B) Immunohistochemical study revealed that BMP-6 protein is expressed in the adrenal cortex derived from 12-week-old male WKY rats and age-matched spontaneously hypertensive rats (SHR). It is of note that BMP-6 protein expression was densely detected in the zona gromerulosa of adrenal cortex of SHR. Bars indicate 30 μm in size.

Fig. 10. **Possible involvement of BMP-6 in aldosterone breakthrough in adrenocortical cells.** 1) BMP-6 signaling supports Ang II-induced aldosterone synthesis by sustaining Ang II-induced ERK1/2 phosphorylation. 2) Cellular expression of BMP-6, the receptor (ALK-2 and ActRII) and Smad1,5,8 activation were downregulated by long-term Ang II exposure. 3) However, in the presence of co-treatment with ARB, the activation of BMP-6 signaling is maintained, which may lead to aldosterone breakthrough phenomenon *in vitro*.

Fig. 1

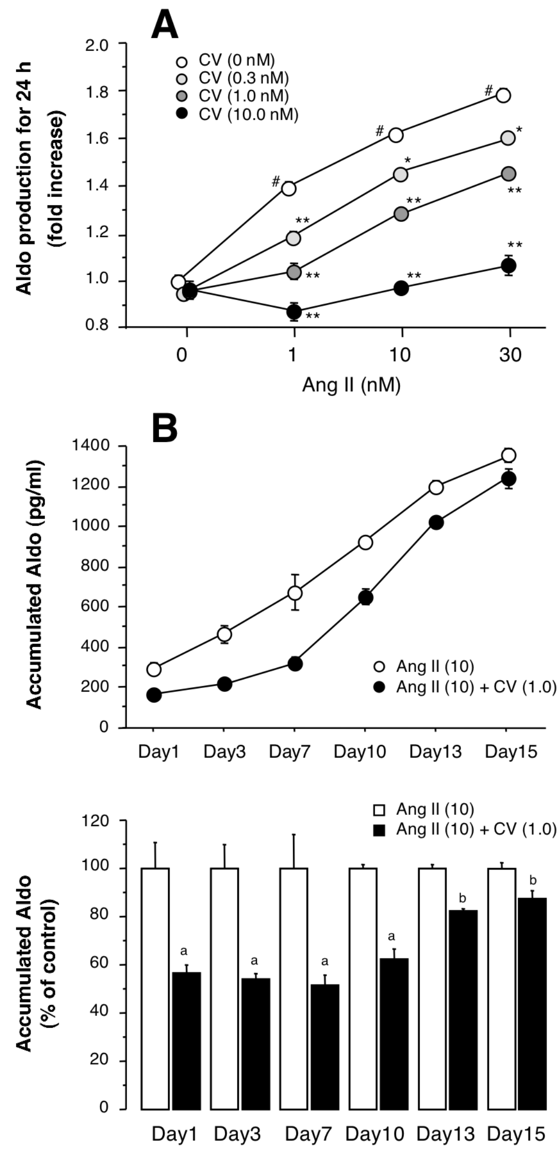
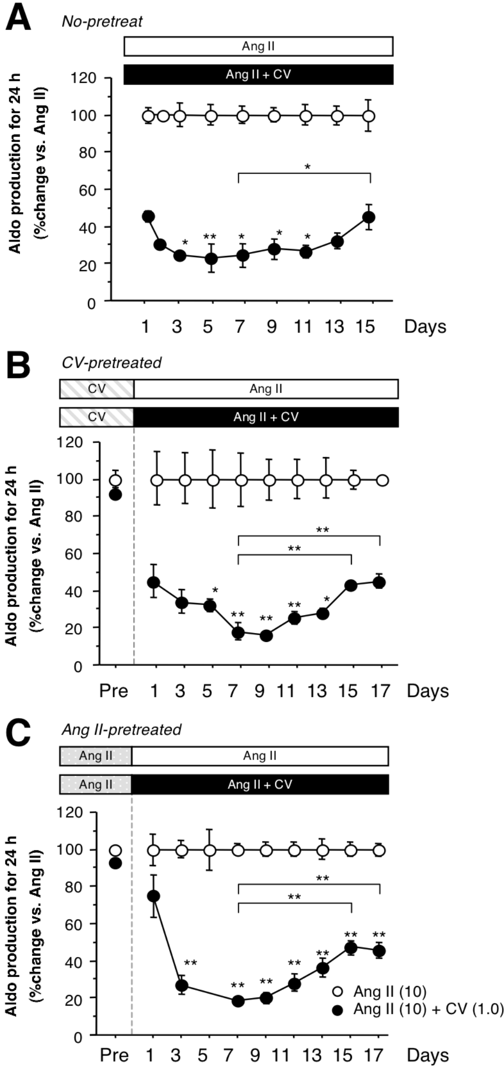


Fig. 2



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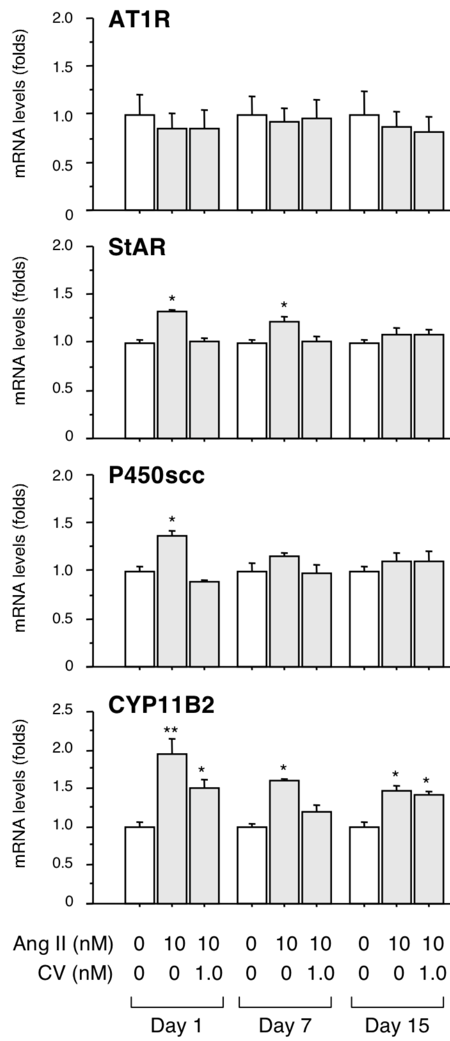

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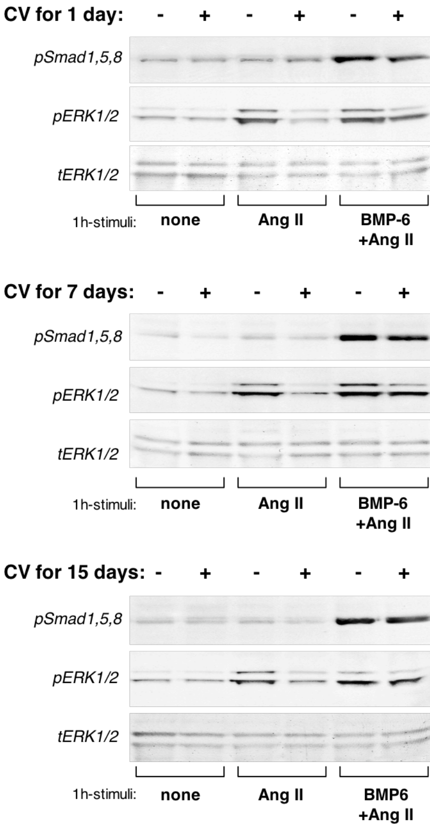
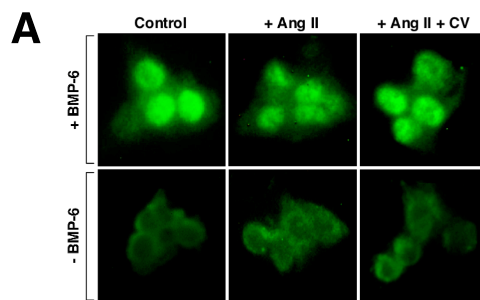


Fig. 5



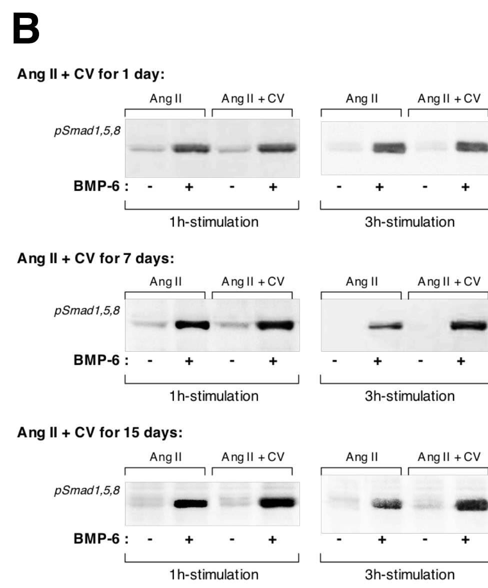
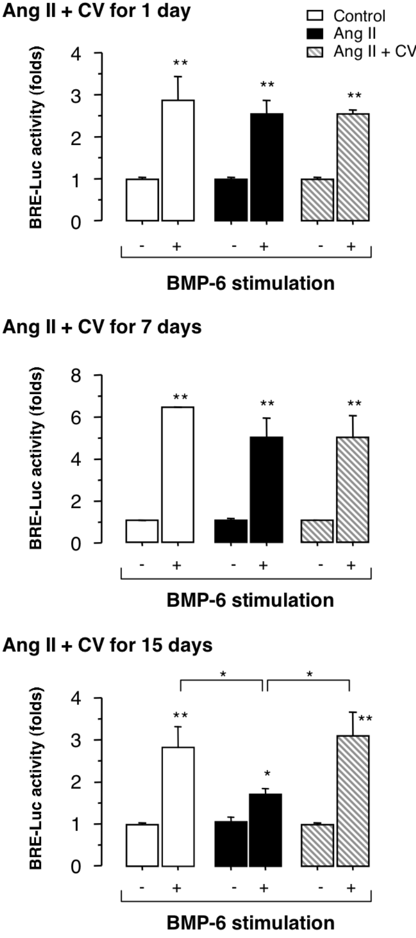


Fig. 6



New **Fig. 7**

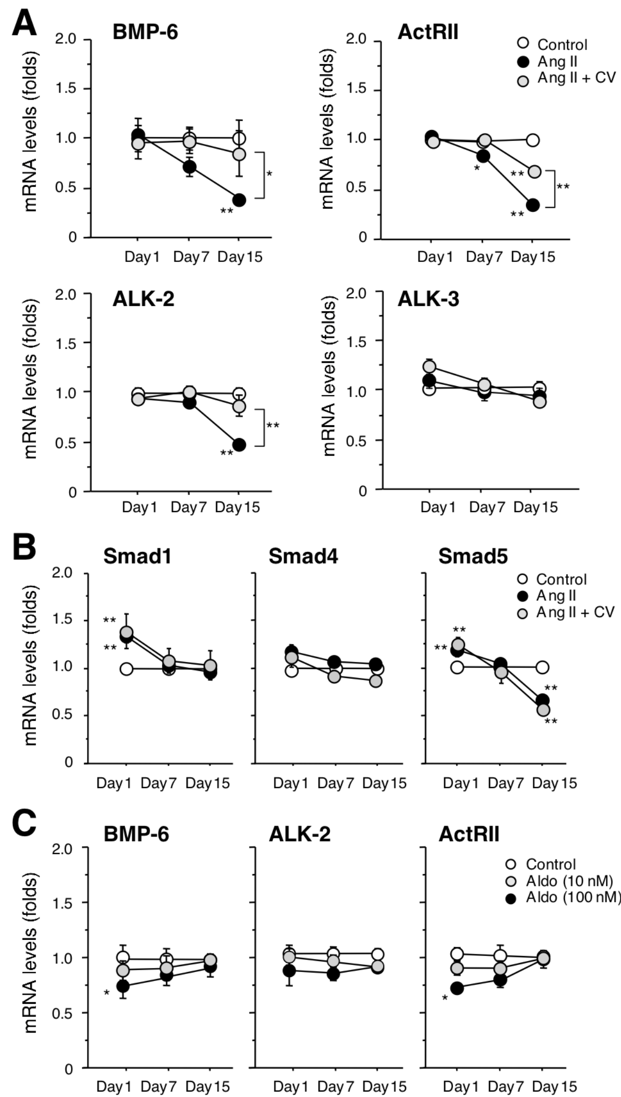
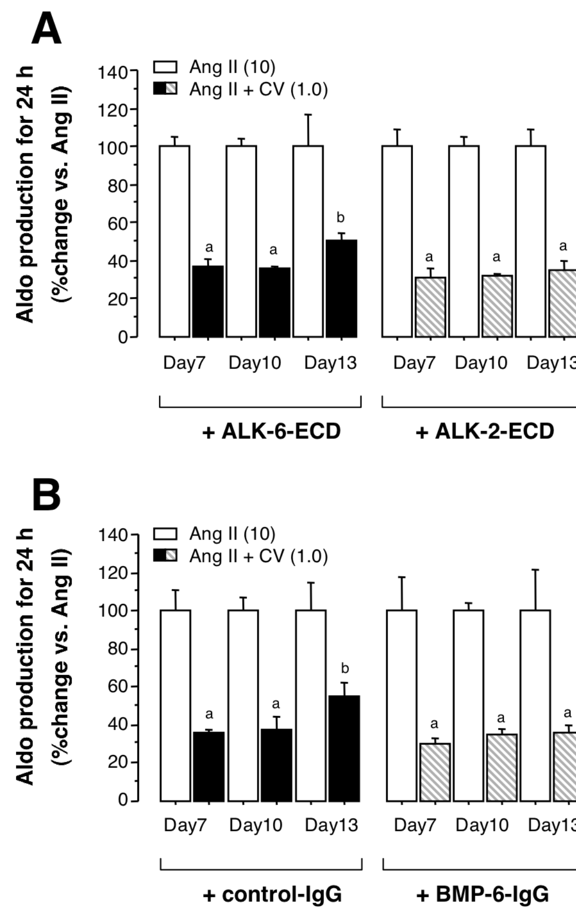


Fig. 8



New Fig. 9

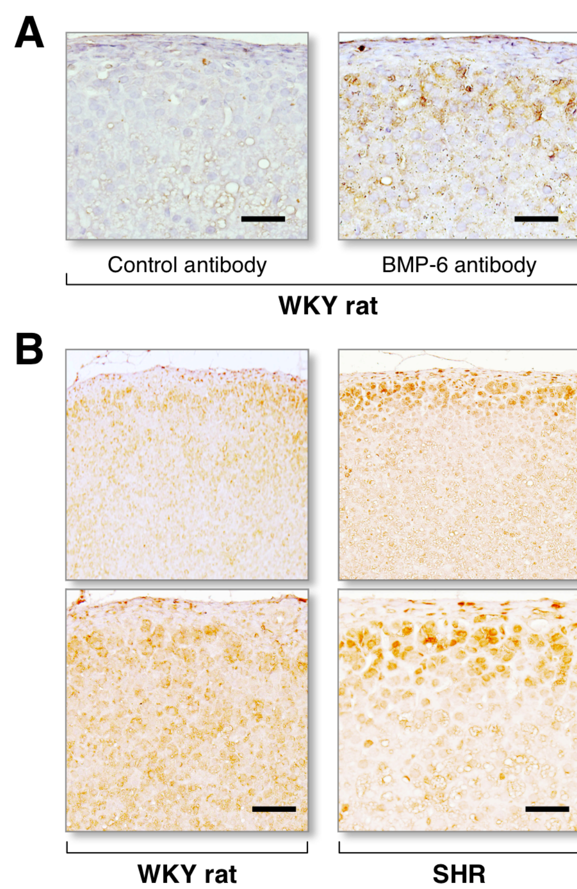


Fig. 10

3) Blockage of Ang II signaling may fail to downregulate the BMP-6 system.

2) Long-term Ang II signaling inhibits adrenocortical BMP-6 system.

Ang II

ARB

AT1R

ERK

Steroidogenic Enzymes

Aldosterone

BMP-6

ALK-2

ActRII

Smad1,5,8

1) BMP-6 signaling supports Ang II-induced ERK pathway leading to maintenance of Aldo production.