Enhancement of aldosterone-induced catecholamine production by bone morphogenetic protein-4 through activating Rho and SAPK/JNK pathway in adrenomedullar cells

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Running title: Aldosterone and BMP-4 on catecholamine biosynthesis

Key words: Aldosterone, Bone morphogenetic protein, Catecholamines, Eplerenone, Mineralocorticoid receptor, Pheochromocytoma

Disclosure statement: All authors have nothing to disclose.

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Abbreviations:
ALK, activin receptor-like kinase; ActRII, activin type-II receptor; BMP, bone morphogenetic protein; BMPRII, BMP type-II receptor; DBH, dopamine-β-hydroxylase; DDC, 3,4-dihydroxyphenylalanine decarboxylase; DOPA, 3,4-dihydroxyphenylalanine; 11βHSD2, 11 beta-hydroxysteroid dehydrogenase type 2; ERK, extracellular signal-regulated kinase; GR, glucocorticoid receptor; IBMX, 3-isobutyl-1-methyl-xanthine; MAPK, mitogen-activated protein kinase; MR, mineralocorticoid receptor; SAPK/JNK, stress-activated protein kinase / c-Jun NH2-terminal kinase; TGF-β, transforming growth factor-β; TH, tyrosine hydroxylase

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ABSTRACT

Here we investigated the effects of mineralocorticoid in the regulation of catecholamine biosynthesis using rat pheochromocytoma PC12 cells. Expression of mineralocorticoid receptor (MR) was confirmed in undifferentiated PC12 cells. Aldosterone stimulated dopamine production by PC12 cells without any increase in cAMP activity. Aldosterone-induced dopamine accumulation was enhanced in accordance with the increase in the rate-limiting enzyme, tyrosine hydroxylase (TH). Blocking MR with eplerenone suppressed aldosterone-induced increases of TH mRNA and dopamine production. A glucocorticoid receptor antagonist, RU486, attenuated dexamethasone-but not aldosterone-induced TH expression. Cycloheximide reduced both aldosterone- and dexamethasone-induced TH mRNA. A SAPK/JNK inhibitor, SP600125, suppressed aldosterone-induced TH mRNA expression; however, the aldosterone-induced TH expression was not affected by inhibition of ERK1/2, p38-MAPK, Rho-kinase, PI3-kinase and PKC. It was of note that co-treatment with eplerenone and SP600125 restored aldosterone-induced TH mRNA expression to basal levels. To investigate the involvement of bone morphogenetic protein (BMP) actions in aldosterone-induced catecholamine production, we examined the effects of BMP-4 and BMP-7, which are expressed in the adrenal medulla, on catecholamine biosynthesis. BMP-4 preferentially enhanced aldosterone-induced TH mRNA and dopamine production although BMP-4 alone did not affect TH expression. The BMP-4 enhancement of aldosterone-induced TH expression was not observed in cells treated with eplerenone. BMP-4 did not affect MR expression of PC12 cells; however it did enhance aldosterone-induced SAPK/JNK phosphorylation. Inhibition of SAPK/JNK or
Rho suppressed BMP-4 enhancement of aldosterone-induced TH expression. Collectively, our findings demonstrate that aldosterone stimulates catecholamine biosynthesis in adrenomedullar cells via MR through genomic action and partly through nongenomic action by Rho-SAPK/JNK signaling, the latter of which is facilitated by BMP-4. A functional link between MR actions and endogenous BMP may be involved in the catecholamine production.
INTRODUCTION

Accumulating evidence has shown that the adrenal cortex and medulla functionally interact with each other in a paracrine manner due to their close embryological structure (6, 54). Endogenous glucocorticoids are well known to induce catecholamine biosynthesis by stimulating catecholamine-synthesizing enzymes through the cortico-medullary portal system (69). We earlier reported the presence of bone morphogenetic protein (BMP) system in the adrenal and a functional crosstalk between glucocorticoid and BMP system in regulating catecholamine synthesis in adrenomedullar cells (32).

BMPs belong to the transforming growth factor-β (TGF-β) superfamily and have been demonstrated to exert a variety of physiological actions in various endocrine organs, including ovary (56), pituitary (45, 49, 64), thyroid (62), and adrenal (28, 32, 61). It has been shown that key components of the BMP system are expressed throughout neural development in complex patterns for fine-tuning various developmental stages in an autocrine and/or paracrine manner (41). BMP-4 and BMP-7 are expressed in the dorsal aorta and direct sympathetic neuronal differentiation into the adrenergic characteristics (51). Furthermore, BMP-4 and BMP-7 induce tyrosine hydroxylase (TH)-immunoreactive adrenergic phenotype in cultures of avian neural crest cells (67, 68). However, the physiological roles of BMPs in regulation of neuroendocrine dynamics after phenotypic maturation of sympathoadrenal neurons remain to be elucidated.

As for the interrelationship between aldosterone and dopamine in the adrenal, control of adrenal aldosterone production by dopamine is widely recognized and
established (9, 20, 21). A dopamine antagonist stimulates aldosterone secretion, which can, in turn, be blocked by dopamine. Dopamine also inhibits angiotensin II- and potassium-induced aldosterone secretion from bovine adrenal cells (15, 40). The dopamine actions suppressing aldosterone are likely to occur via the dopamine D2 receptor (44), although adrenal cortex expresses both D1 and D2 receptors (2, 4, 5). The dopamine D1 receptor appears to be linked to the stimulation of adenylate cyclase, whereas the D2 receptor is coupled in an inhibitory manner to cAMP synthesis (43).

The effects of aldosterone on neighboring chromaffin cells; however, remain poorly understood although mineralocorticoids are major hormones produced from adrenal cortex. Recent studies have shown that aldosterone exerts various biological actions not only by classical genomic actions through regulation of nuclear gene transcription and protein synthesis via MR but also by rapid, potentially nongenomic responses (17). In epithelial cells, MR is protected from excessive cortisol by co-expressing 11 beta-hydroxysteroid dehydrogenase type 2 (11βHSD2) which converts active cortisol to inactive cortisone in vivo (14, 18), whereas in non-epithelial tissues without expression of 11βHSD2, MR is recognized to be occupied by overwhelmingly existing cortisol rather than aldosterone in vivo. Given the finding that patients with primary aldosteronism exhibit higher plasma dopamine levels compared with essential hypertensive patients (27, 33), the possible influence of aldosterone on adrenal medullar functions can be speculated.

In the present study, we investigated aldosterone actions on catecholamine biosynthesis utilizing rat adrenomedullar cell line PC12. The PC12 cell line is a clone of cells derived from a rat adrenal medullary tumor. PC12 cells exhibit many properties of adrenal medullary chromaffin cells including catecholamine synthesis, storage and
secretion (24). Catecholamine biosynthesis in which tyrosine is converted into dopamine is initially catalyzed by TH to produce 3,4-dihydroxyphenylalanine (DOPA). DOPA is then converted to dopamine by DOPA decarboxylase (DDC), also called aromatic L-amino acid decarboxylase. Dopamine-β-hydroxylase (DBH) converts dopamine to noradrenaline. Since the expression of MR was confirmed in rat pheochromocytoma-derived undifferentiated PC12 cells, we here investigated the aldosterone-induced actions involved in the catecholamine production by PC12 cells. A possible functional crosstalk between aldosterone and BMP system in regulation of catecholamine production was uncovered.
MATERIALS AND METHODS

Reagents and Supplies

Dulbecco’s Modified Eagle’s Medium (DMEM), penicillin-streptomycin solution, d-aldosterone, cycloheximide (CHX), dimethylsulfoxide (DMSO), dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), Clostridium difficile Toxin B and geranylgeranyl transferase I inhibitor (GGTI)-298 were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Recombinant human BMP-4 and BMP-7 were purchased from R&D Systems, Inc. (Minneapolis, MN). U0126, SB203580, Myristoylated Protein Kinase C Peptide Inhibitor (PKCI) and LY294002 were purchased from Promega Corp. (Madison, WI), SP600125 was from Biomol Lab. Inc. (Plymouth Meeting, PA), and Y-27632 was from Calbiochem (San Diego, CA). Eplerenone was provided by Pfizer Inc. (New York, NY). Pioglitazone and fenofibric acid were provided from Takeda Chemical Industries (Osaka, Japan) and Kaken Pharmaceutical Co. Ltd. (Tokyo, Japan), respectively. PC12 cells were kindly provided by Dr. Isao Date, Department of Neurosurgery at Okayama University, Japan. Normal rat adrenal tissues were obtained from male Sprague-Dawley rats, spontaneously hypertensive rats and Wistar-Kyoto rats (Charles-River Lab, Wilmington, MA). The protocol was conducted in accordance with an animal use protocol approved by our institutional committee.

Cell culture and catecholamine analysis

PC12 cells were maintained in DMEM supplemented with 10% fetal calf serum (FCS), 10% horse serum (HS), penicillin and streptomycin (Sigma-Aldrich Corp.) at 37°C in 5% CO₂ humidified atmosphere. The culture medium was changed twice a week, and
cultures were passaged at ~80% confluence. Changes in cell morphology were monitored after treatments with aldosterone, dexamethasone, pioglitazone and fenofibric acid using an inverted microscope using chamber slides (Nalge Nunc Int., Naperville, IL). PC12 cells (1 × 10^5 viable cells) were precultured in DMEM containing 1% FCS and 1% HS for 24 h and then were treated with the indicated concentrations of aldosterone, dexamethasone in combination with a selective MR antagonist eplerenone, BMP-4 and BMP-7. The culture medium was collected after 24-h culture and the catecholamine levels including dopamine, noradrenaline and adrenaline were determined by high-performance liquid chromatography (HPLC; Tosoh Analysis and Research Center Co., Shunan, Japan).

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

PC12 cells (3 × 10^5 viable cells) were precultured in DMEM containing 1% FCS and 1% HS in 12-well plates, and then cells were treated with indicated concentrations of aldosterone, dexamethasone, BMP-4 and BMP-7, in combination with a selective MR antagonist eplerenone, a GR antagonist RU486, and various chemical inhibitors including cycloheximide, U0126, SB203580, SP600125, PKCI, LY294002 and Y-27632. After 24-h culture, the medium was removed, and total cellular RNAs were extracted using TRIzol® (Invitrogen Corp.), subsequently quantified by measuring absorbance at 260 nm and stored at -80°C until assay. Total RNA of adrenal tissues was also extracted using TRIzol® from 7-week-old male Sprague-Dawley rats. The expression of MR, GR, catecholamine synthases (TH, DDC and DBH), 11βHSD2, BMP ligands, BMP receptors and Smads was detected by RT-PCR analysis. 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) at 42°C for 50 min, 70°C for 10 min. Subsequently, hot-start PCR was performed using MgCl2 (1.5 mM), dNTP (0.2 mM) and 2.5 U of Taq DNA polymerase (Invitrogen Corp.). Oligonucleotides used for 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. The primer pairs for rodent BMP ligands, BMP receptors, Smads, a house-keeping gene ribosomal protein-L19 (RPL19) and catecholamine synthase genes including TH, DDC and DBH were selected as we reported (32, 45, 63). For GR, MR and 11βHSD2 genes, the following sequences were used: GR, 1895-1915 and 2171-2191 from GenBank accession number X04435; MR, 2978-2999 and 3219-3240 from M36074; and 11βHSD2, 622-642 and 891-911 from NM_017081. The PCR product sizes are as follows: BMP-4, 241 bp; BMP-7, 287 bp; ALK-2, 483 bp; ALK-3, 510 bp; ALK-6, 456 bp; BMPRII, 522 bp; ActRIIA, 492 bp; ActRIIB, 271 bp; TH, 445 bp; DDC, 387 bp; DBH, 364 bp; GR, 297 bp; MR, 263 bp; 11βHSD2, 290 bp; and RPL19, 195 bp. The aliquots of PCR products were electrophoresed on 1.5% agarose gels and visualized after ethidium bromide staining. For the quantification of TH, MR, BMP-4, BMP-7 and RPL19 mRNA levels, real-time PCR was performed using LightCycler-FastStart DNA master SYBR Green I system® (Roche Diagnostic Co., Tokyo, Japan) under conditions of annealing at 60°C with 4 mM MgCl2, following the manufacturer’s protocol. Accumulated levels of fluorescence were analyzed by the second derivative
method after the melting-curve analysis (Roche Diagnostic Co.) and then the expression levels of target genes were standardized by RPL19 level in each sample.

**Measurement of cAMP production**

To assess effects of aldosterone and dexamethasone on cAMP synthesis, PC12 cells (1 × 10^5 viable cells) were cultured in 96-well plates with DMEM containing 10% FCS and 10% HS for 24 h. The medium was then changed to DMEM containing 1% FCS and 1% HS, and subsequently treated with the indicated concentrations of dexamethasone and aldosterone in the presence of 0.1 mM IBMX (a specific inhibitor of phosphodiesterase activity). After 24-h culture, the supernatant of the culture media was collected and stored at -80°C until assay. The extracellular contents of cAMP were determined by enzyme immunoassay (Assay Designs, Inc., Ann Arbor, MI) after the acetylation of each sample with assay sensitivity of 0.039 nM.

**Western immunoblot analysis**

PC12 cells (1 × 10^5 viable cells) were precultured in 12-well plates in serum-free DMEM. After 24-h preculture, aldosterone (100 nM) was added to the culture medium either alone or in combination with SP600125 (10 µM) and BMP-4 (100 ng/ml). After 10-, 30- and 60-min stimulation with hormones and/or growth factors, cells were solubilized in 100 µl RIPA lysis buffer (Upstate Biotechnology, Lake Placid, NY) containing 1 mM Na_3VO_4, 1 mM NaF, 2% SDS and 4% β-mercaptoethanol. After stimulation with hormones and/or growth factors for indicated periods, the membrane fraction of PC12 cells was extracted by ProteoExtract® Native Membrane Protein
Extraction Kit (Calbiochem, San Diego, CA). The cell lysates and the membrane fractions were then subjected to SDS-PAGE/immunoblotting analysis as we previously reported (28, 48), using anti-phospho- and anti-total-stress-activated protein kinase (SAPK)/c-Jun NH₂-terminal kinase (JNK) antibody (Cell signaling Technology, Inc., Beverly, MA), anti-phospho-Smad1,5,8 antibody (Cell signaling Technology, Inc.), anti-MR antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-RhoA antibody (Santa Cruz Biotechnology, Inc.), and anti-actin antibody (Sigma-Aldrich Corp.). For immunoblot analysis of MR detection, a positive control from K-562 whole cell lysate (Santa Cruz Biotechnology, Inc.) was utilized.

**Immunofluorescence microscopy**

For immunofluorescence study, PC12 cells were precultured in serum-free DMEM using chamber slides (Nalge Nunc Int.). Cells at ~50% confluency were treated with BMP-4 (100 ng/ml) for 1 h. Cells were then fixed with 4% paraformaldehyde 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 obtained from 12-week-old male spontaneously
hypertensive rats and Wistar-Kyoto rats were dewaxed and rehydrated, and antigen retrieval was performed by heating for 10 min in a 10 mM citrate buffer at pH 6.0. The sections were reacted with gout polyclonal antibody for BMP-4 (R&D Systems, Inc.) diluted at 15 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

We first confirmed the mRNA expression of catecholamine synthases including a rate-limiting enzyme TH, DDC and DBH by RT-PCR in PC12 cells as well as in the rat whole adrenals and medullar tissues (Fig. 1A). PC12 cells expressed MR and GR as did the whole adrenal and adrenomedullar tissues. In addition, 11βHSD2 expression was also detected in PC12 cells and in rat whole adrenals and medullar tissues (Fig. 1A). The protein expression of MR was further confirmed by immunoblotting analysis in undifferentiated PC12 cells. The expression of MR protein (~107 kDa) was detected in PC12 cells treated with dexamethasone (1 µM), aldosterone (100 nM) and a PPARα agonist, fenofibric acid (1 µM) (Fig. 1B). However, the MR protein expression was nearly undetectable in differentiated PC12 cells treated with a PPARγ agonist, pioglitazone (1 µM). No specific differences of the morphological changes indicating neural differentiation were shown between PC12 cells treated with dexamethasone (1 µM), aldosterone (100 nM) and fenofibric acid (1 µM). However, PC12 cells treated with a PPARγ agonist, pioglitazone (1 µM), showed a marked differentiation with typical neurite outgrowth (16). The expression levels of internal control actin (42 kDa) were not changed regardless of the treatment (Fig. 1B). To investigate the involvement of BMP actions in aldosterone-induced catecholamine production, we examined the presence of key components of the BMP system including BMP ligands, BMP type-I and type-II receptors, and Smads in PC12 cells and rat adrenal tissues. As shown in Fig. 1C, PC12 cells and adrenal medulla clearly expressed BMP-4, -7, ALK-2, -3, ActRIIA, ActRIIB, BMPRII, and receptor-regulated Smads1, 2,
3, 5 and 8, common Smad4, and inhibitory Smads6 and 7. Furthermore, BMP-4 protein expression was detected by immunohistochemical analysis in rat adrenomedullar tissues (Fig. 1D).

We next studied effects of aldosterone or dexamethasone on catecholamines biosynthesis in PC12 cells. Dopamine secretion attained to ~200 pg/ml in the conditioned medium during 24-h culture, although the secreted levels of noradrenaline and adrenaline by PC12 cells were not attained to detectable ranges. Based on our earlier optimization regarding the determination of catecholamine production by undifferentiated PC12 cells, PC12 cells were cultured in media containing 1% FCS and 1% HS (32). Culturing PC12 cells in media containing 1% serum attained the highest dopamine secretion into the media with arrested cell proliferation. Time-course experiments revealed that dopamine levels saturate after 48-h culture and the least variable at the 24-h time point. In this culture condition, aldosterone and dexamethasone increased dopamine production in a concentration-responsive manner in accumulated medium (Fig. 2A). In accordance with the increase in dopamine, mRNA of a rate-limiting enzyme for catecholamine production TH was upregulated by aldosterone and dexamethasone (Fig. 2B). However, accumulated levels of cAMP were not affected by aldosterone or dexamethasone, indicating that cAMP signaling is not directly involved in catecholamine biosynthesis by mineralocorticoids and glucocorticoids in PC12 cells (Fig. 2C).

The involvement of MR/GR in regulating TH induction was examined in order to elucidate the mechanisms by which aldosterone induces dopamine production. Eplerenone, a selective MR antagonist, significantly suppressed aldosterone- but not dexamethasone-induced TH mRNA expression (Fig. 3A). Aldosterone (100 nM)-
induced TH mRNA levels were reduced by 15% and 35%, in the presence of 100 nM and 300 nM of eprelenone, respectively. In contrast RU486, a GR antagonist, preferentially decreased dexamethasone-induced TH mRNA levels (Fig. 3B). A translational inhibitor, cycloheximide (CHX) also decreased aldosterone- and dexamethasone-induced TH mRNA levels (Fig. 3C). In addition, the inhibitory effect of eplerenone on aldosterone-induced TH expression was not significantly affected by co-treatment with RU486 (Fig. 3D). Likewise, the effects of RU486 on dexamethasone-induced TH mRNA were not affected by addition of eplerenone (Fig. 3E).

There has been accumulating evidence showing that aldosterone also induces rapid activation of intracellular signaling pathways including mitogen-activated protein kinase (MAPK) (22, 25, 48), Rho-kinase (60), PI3-kinase (PI3K) (39) and protein kinase C (PKC) (11, 36), in a cell-specific manner. To study the functional involvement of the intracellular signaling in PC12 cells, we examined inhibitory effects of MAPK, Rho-kinase, PI3K and PKC pathways on aldosterone-induced TH mRNA expression. As shown in Fig. 4, U0126 and SB203580, inhibitors for ERK1/ERK2 and p38-MAPK pathway, respectively, did not affect either aldosterone- or dexamethasone-induced TH mRNA levels. Notably, a SAPK/JNK inhibitor, SP600125 suppressed aldosterone-induced TH mRNA expression in a concentration-dependent manner. In contrast, dexamethasone-induced TH expression was not affected by SP600125 (Fig. 4C). A Rho-kinase inhibitor Y-27632, a PI3K inhibitor LY294002, and a PKC inhibitor (PKCI) had no significant effects on aldosterone- or dexamethasone-induced TH mRNA levels. These data suggest that SAPK/JNK signaling is potentially involved in aldosterone-induced TH expression and catecholamine production. Furthermore, treatment with
eplerenone (Fig. 5A) or cycloheximide (Fig. 5B) in combination with a SAPK/JNK inhibitor, SP600125, restored aldosterone-induced TH mRNA expression to the basal levels. Likewise, eplerenone in combination with various concentrations of SP600125 restored aldosterone-induced dopamine production (Fig. 5C), suggesting that both of MR-mediated genomic actions and SAPK/JNK pathway are functionally involved in regulating aldosterone-induced catecholamine production.

We next examined functional roles of BMP-4 and BMP-7 in catecholamine production by PC12 cells. BMP-4 and BMP-7 by alone had no significant effects on TH mRNA levels (Fig. 6A). As shown in Fig. 6B, BMP-4 preferentially enhanced aldosterone-induced TH mRNA expression as compared to that induced by dexamethasone. In contrast, BMP-7 had no significant effects on aldosterone- and dexamethasone-induced TH mRNA (Fig. 6C). BMP-4 enhancement of aldosterone-induced dopamine production was significantly higher than that induced by BMP-7 (Fig. 7A). In addition, BMP-4 and BMP-7 did not affect the expression levels of MR mRNA and MR protein (Fig. 7B and 7C). Nuclear localization of phosphorylated Smad1,5,8 was demonstrated in PC12 cells stimulated with BMP-4 (Fig. 7D). Furthermore, Smad1,5,8 phosphorylation induced by BMP-4 has preserved regardless of the treatments with aldosterone and dexamethasone (Fig. 7D). Also, the expression of BMP-4 was not affected by the presence of aldosterone or dexamethasone. In contrast, BMP-7 mRNA was reduced in aldosterone-treated PC12 cells (Fig. 7E). These data imply that endogenous BMP-4 and BMP-4-induced Smad signaling are preserved in the process of aldosterone-induced catecholamine production. Importantly, the BMP-4 enhancement of aldosterone-induced TH expression (Fig. 8A) and dopamine production
(Fig. 8B) was abrogated by eplerenone treatment, implying that BMP-4 preferentially facilitates aldosterone actions mediated via MR by PC12 cells.

Next, effects of BMP-4 on aldosterone-induced SAPK/JNK activation were investigated. Aldosterone activated SAPK/JNK phosphorylation during 10- to 60-min observation in PC12 cells, in which SAPK/JNK activation was highest at 30 min after the stimulation (Fig. 9A). Aldosterone significantly induced p54-SAPK/JNK phosphorylation in PC12 cells, which was further enhanced by BMP-4 treatment (Fig. 9A). The p54-SAPK/JNK activation induced by aldosterone, as well as that induced by aldosterone in combination with BMP-4, was significantly suppressed in the presence of SP600125. The p46-SAPK/JNK phosphorylation increased in response to the addition of BMP-4. Aldosterone enhanced the BMP-4-induced p46-SAPK/JNK activation in PC12 cells (Fig. 9A). Thus, aldosterone in the presence of BMP-4 action preferentially stimulated the p54/p46-SAPK/JNK pathway in PC12 cells. Furthermore, involvement of activation of small GTP-binding protein RhoA, an upstream activator for SAPK/JNK pathway (57), was examined in membrane protein fractions of PC12 cells (Fig. 9B). Aldosterone and dexamethasone both induced RhoA activation, i.e., membrane localization of RhoA protein. BMP-4 preferentially enhanced RhoA activation induced by aldosterone as compared to that induced by dexamethasone.

We therefore studied roles of the Rho-SAPK/JNK pathway in the BMP-4 enhancement of aldosterone-induced catecholamine synthesis by PC12 cells (Fig. 10). As shown in the left panel of Fig. 10, SP600125 suppressed BMP-4 enhancement of aldosterone-induced TH mRNA levels, indicating that BMP-4 enhances aldosterone-induced catecholamine production through activating SAPK/JNK signaling. Clostridium difficile Toxin B, which inactivates membrane Rho proteins by
glycosylation modification (31), inhibited the BMP-4 enhancement of aldosterone-induced TH expression (middle panel of Fig. 10), suggesting the functional involvement of Rho proteins in activating TH expression by aldosterone and BMP-4. However, GGTI-298, a geranylgeranyl transferase inhibitor of Rho proteins (37), failed to suppress the BMP-4 enhancement of aldosterone-induced TH expression (right panel of Fig. 10), suggesting that BMP-4 possibly stimulates Rho-SAPK/JNK pathway at the downstream process of geranylgeranylation of cytosolic Rho proteins. Taken together, these findings demonstrate that BMP-4 facilitates MR-mediated actions through activating Rho-SAPK/JNK pathway leading to augmentation of aldosterone-induced TH expression and catecholamine production (Fig. 11).
DISCUSSION

In the present study, we demonstrated that not only glucocorticoids but also mineralocorticoids are involved in regulation of catecholamine biosynthesis in undifferentiated PC12 cells by stimulating dopamine production through the upregulation of TH expression. In adrenomedullary cells, TH is considered to be the rate-limiting enzyme in catecholamine biosynthesis (35), in which TH activity can be governed by acute and chronic regulatory mechanisms (70). Acute regulation of TH activity occurs at a posttranscriptional level mainly through the phosphorylation of TH, resulting in activation of the preexisting enzymes. Chronic activation, which can last from minutes to days, is governed through the regulation of TH transcription. Activity of both TH and DBH is regulated by second messenger mechanisms involving the activation of cAMP, PKA and PKC in PC12 cells (34). However, the present results suggest that aldosterone-induced TH expression and catecholamine production are independent of activation of the cAMP-PKA pathway.

There have been no detailed studies investigating the expression of MR in adrenomedullar cells. PC12 cells have been widely utilized to investigate adrenal catecholamine biosynthesis, chromaffin cell proliferation and differentiation. We here uncovered the expression of MR in undifferentiated stages of PC12 cells lacking neurite outgrowth and rat adrenal medullar tissues. MR activation in PC12 cells plays a pivotal role in the upregulation of TH expression, since a selective MR antagonist, eplerenone, suppressed aldosterone-induced TH expression. Eplerenone inhibition of aldosterone-induced TH expression was not further influenced by co-treatment with RU486. Likewise, RU486 effects on dexamethasone-induced TH mRNA were not affected by
adding eplerenone. Considering the differences in the maximal effects and EC<sub>50</sub> of dopamine induction between aldosterone and dexamethasone, it is presumable that a divergence in the mechanism of GR and MR actions underlies in the control of TH expression and dopamine synthesis by PC12 cells. Considering the fact that aldosterone-induced TH expression was also repressed by a translational inhibitor cycloheximide, genomic MR actions must be functionally involved in modulating TH expression.

In aldosterone-target cells, 11<sub>β</sub>HSD2 plays crucial roles in conferring aldosterone sensitivity on MR by inactivating intracellular glucocorticoids before they occupy MR. It is known that 11<sub>β</sub>HSD2 mRNA and protein are expressed in sheep, rat and rabbit adrenals (1, 47, 52, 58), albeit not in mouse adrenal (12). Roland and Funder also demonstrated the scattered expression of 11<sub>β</sub>HSD2 mRNA throughout rat adrenal with relatively abundant signal in the zona fasciculata and weak signal in the medulla (52). 11<sub>β</sub>HSD2 may protect adrenomedullar cells from excessive effects of glucocorticoids on catecholamine induction. The presence of 11<sub>β</sub>HSD2 in the adrenal medulla and PC12 cells also supports our hypothesis that adrenomedullar MR is functionally involved in regulating catecholamine production.

In addition to the genomic actions of aldosterone mediated through regulation of nuclear gene transcription and protein synthesis, aldosterone also elicits rapid, potentially nongenomic, responses including intracellular calcium, PKC, MAPK, Rho-kinase, and PI3K in a variety of cells (17, 55). In this regard, classical MR primarily acts as a transcription factor; however, recent evidence has shown that MR may also mediate nongenomic activation of various second-messenger pathways (26). For
instance, MR antagonists block aldosterone-induced activation of ERK1/2 in vascular smooth muscle cells (30) and Chinese hamster ovary cells transfected with human MR (25), whereas it is also reported that aldosterone actions on ERK1/2 activation were unaffected by MR antagonists in cortical collecting duct cells (53). MR antagonists enable to attenuate the rapid aldosterone-induced activation of Ki-RasA (59) and c-Src (8) which leads to MAPK activation in cardiovascular cells. MR antagonists are also able to block several nongenomic actions of aldosterone on vascular Na⁺/K⁺-ATPase (3) and arterial tone (39, 42, 65). Hence the interaction between MR and its nongenomic responses is still controversial.

In this study, aldosterone induced a rapid phosphorylation of the SAPK/JNK pathway. Aldosterone induced p54-SAPK/JNK phosphorylation in PC12 cells, which was further enhanced by BMP-4 treatment. The p46-SAPK/JNK phosphorylation was upregulated by BMP-4 treatment, which was further enhanced by aldosterone action. Thus, aldosterone in combination with BMP-4 stimulates p54/p46-SAPK/JNK pathway in PC12 cells. Mammalian SAPK/JNK proteins are encoded by three genes including \textit{jin1}, \textit{jin2} and \textit{jin3}. The \textit{jin1} and \textit{jin2} genes are ubiquitously expressed but \textit{jin3} gene is expressed primarily in the brain. The \textit{jin} genes are spliced alternatively to create 10 isoforms, in which spliced variants at the C-terminus leads to proteins that are either p46 (SAPK/JNK1) or p54 (SAPK/JNK2) in size (29). Similar to other MAP kinase pathways, the JNKs require phosphorylation via an upstream kinase cascade to become active. The SAPK/JNK group is strongly activated by proinflammatory cytokines or extracellular stresses such as irradiation and heat shock and plays important roles in regulating various cellular functions including proliferation, development, and apoptosis (13). Both SAPK/JNK1 and SAPK/JNK2 contribute to cellular SAPK/JNK activity in
response to various stimuli, and these proteins not only have shared biologic functions but also elicit distinct or even opposing biologic functions (38, 66).

Since the inhibition of SAPK/JNK pathway suppressed aldosterone-induced TH expression, rapid nongenomic effects of aldosterone mediated by SAPK/JNK signaling seem functionally involved in the TH expression. Involvement of SAPK/JNK and PKC pathways in catecholamine biosynthesis is also reported in PC12 cells treated with prolactin-releasing peptide (46). Given the finding that co-treatment of either eplerenone or cycloheximide in combination with SP600125 restored aldosterone-induced TH expression to the basal levels, both of MR-mediated genomic actions and nongenomic actions through SAPK/JNK signaling appeared to be linked to aldosterone-induced catecholamine production.

We previously reported that glucocorticoid and endogenous BMP system are functionally involved in regulating catecholamine biosynthesis in adrenomedullar cells (32). Other studies have also demonstrated that BMPs regulate sympathetic neuronal differentiation into the adrenergic phenotype (51), in which BMP-4 and BMP-7 induce TH-immunoreactive adrenergic phenotype in neural crest cells (68). However, the physiological roles of BMPs in regulation of neuroendocrine dynamics after phenotypic maturation of sympathoadrenal neurons remain to be elucidated. In the present study, it was of note that both BMP-4 and BMP-7 accelerated aldosterone-induced dopamine production. BMP-4 preferentially increased aldosterone-stimulated TH expression although BMP-4 and BMP-7 by alone had no specific effects on TH and MR expression. Eplerenone abolished BMP-4 enhancement of aldosterone-induced dopamine production and TH expression, suggesting that BMP-4 facilitates the actions mediated via MR. In addition, BMP-4 expression in PC12 cells was not downregulated by
aldosterone or dexamethasone, suggesting the bioavailability of endogenous BMP-4 regardless of MR or GR activation. Taken consider our finding that eplerenone also reduced BMP-4 effects on aldosterone-induced activation of SAPK/JNK signaling (data not shown), BMP-4 is most likely to facilitate MR-mediated nongenomic actions through activating SAPK/JNK, leading to augmentation of aldosterone-induced dopamine production and TH expression.

Small GTP-binding proteins exist in an inactive GDP-bound cytosolic form and upon cellular activation they exchange GTP and translocate to the active-membrane form. Posttranslational modification of various small GTP-binding proteins including Ras and Ras-like proteins such as Rho, Rac and Rab (10) is known to interact with the downstream MAPKs and plays a central role in cellular responses such as cell proliferation, differentiation, apoptosis, migration, contraction and regulation of gene transcription (23, 37, 50). Activated Ras/Rho proteins are key components in signal-transducing kinase cascades including MAPKs (57). In particular, RhoA directly binds to the MEK kinase MEKK1, a kinase that activates the ERK and SAPK/JNK pathways (19). MEKK1 acts as a scaffold element for RhoA signaling to SAPK/JNK pathway.

In our study, aldosterone-induced RhoA activation is enhanced by BMP-4 in PC12 cells. The inhibition of Rho activity by Toxin B significantly suppressed TH mRNA levels induced by co-treatment with aldosterone and BMP-4, which mimicked the effect of SAPK/JNK inhibitor SP600125 on reducing TH expression. Therefore, membrane Rho and SAPK/JNK pathway are functionally linked to TH expression induced by aldosterone and BMP-4. The anchoring of small GTP-binding proteins to cell membranes also requires prenylation, in which Rho proteins are modified with geranylgeranylation (37). Given that inhibition of geranylgeranylation by GGTI-298
did not affect the TH expression induced by aldosterone and BMP-4 in the present study, the prenylation process presumably less accounts for the TH upregulation induced by aldosterone and BMP-4 by PC12 cells. Future investigation is necessary to elucidate the underlying molecular interaction by which aldosterone and BMP-4 elicit activation of Rho-to-SAPK/JNK cascade.

Collectively, a novel functional interrelationship between aldosterone and BMP system in regulating catecholamines production in undifferentiated PC12 cells was demonstrated by the present studies (Fig. 11). Aldosterone stimulated dopamine production and TH mRNA expression through MR-mediated genomic actions and partly through nongenomic actions of Rho-to-SAPK/JNK pathways. BMP-4 augmented aldosterone-induced dopamine production and TH expression by upregulating nongenomic actions through rapid activation of Rho-SAPK/JNK pathways, at least in part, via MR. Brown et al. also reported that 11-oxygenated steroids, including aldosterone and dexamethasone, increase intracellular dopamine and noradrenaline levels using primary culture of adrenal pheochromocytoma cells from human subjects (7). Since chromaffin cells are exposed to various hormones produced from neighboring adrenocortical cells within the adrenal, one may consider that not only glucocorticoids but also mineralocorticoids influence hormonal properties of chromaffin cells in relation to this functional crosstalk between MR and endogenous BMP system expressed in adrenomedullar tissues.
ACKNOWLEDGEMENTS

We thank Dr. R. Kelly Moore for helpful discussion and critical reading of the manuscript. This work was supported in part by Grants-in-Aid for Scientific Research, The Ichiro Kanahara Foundation, Kato Memorial Bioscience Foundation, Terumo Lifescience Foundation, and The Naito Foundation.
REFERENCES


FIGURE LEGENDS

Fig. 1. Expression of cathecholamine synthases, MR/GR, 11βHSD2 and BMP system in PC12 cells and adrenal tissues. A) Total cellular RNAs were extracted from PC12 cells, rat whole adrenal and adrenomedullar tissues. Total cellular RNA was quantified by measuring the absorbance of the sample at 260 nm. The expression of mRNAs encoding catecholamine synthases (TH, DDC and DBH), MR, GR, 11βHSD2 and house-keeping gene RPL19 was examined by RT-PCR analysis. Aliquots of PCR products were electrophoresed on 1.5% agarose gel, visualized by ethidium bromide staining and shown as representative of those obtained from three independent experiments. MM indicates molecular weight marker. B) After preculture with DMEM with 1% FCS and 1% HS, PC12 cells were treated with dexamethasone (+Dex; 1 µM), aldosterone (+Aldo; 100 nM), fenofibric acid (+PPARα; 1 µM) and pioglitazone (+PPARγ; 1 µM) for 24 h. Cells were then solubilized and the cell lysates were subjected to Western immunoblotting analysis using anti-MR and anti-actin antibodies. K-562 whole cell lysate was used for a positive control of immunoblot analysis of MR detection. Changes in cell morphology were monitored after each treatment using an inverted microscope using chamber slides. C) The expression of mRNAs encoding BMP ligands (BMP-4 and -7), BMP type-I (ALK-2, -3 and -6) and type-II receptors (ActRIIA, ActRIIB and BMPRII) and Smads (Smad1, 2, 3, 4, 5, 6, 7 and 8) were examined by RT-PCR analysis in PC12 cells, rat whole adrenal and adrenomedullar tissues. Results are shown as representative of those obtained from three independent experiments. MM indicates molecular weight marker. D) The expression of BMP-4
protein was examined by immunohistochemical (IHC) analysis using anti-BMP-4 antibody on adrenomedullar tissue sections. The figures are shown as representative of those obtained from male spontaneously hypertensive rats. Bars indicate 30 µm in size.

**Fig. 2. Effects of aldosterone and dexamethasone on dopamine production and TH expression by PC12 cells.** A) PC12 cells (1 × 10^5 viable cells) were precultured in DMEM containing 1% FCS and 1% HS for 24 h, and then cells were treated with the indicated concentrations of aldosterone (Aldo) or dexamethasone (Dex). The culture medium was collected after 24-h culture and the catecholamine levels were determined by HPLC. B) PC12 cells (3 × 10^5 viable cells) were precultured and treated with the indicated concentrations of Aldo or Dex. After 24-h culture, the medium was removed, and total cellular RNAs were extracted. Steady-state mRNA levels of TH and RPL19 were analyzed by quantitative real-time RT-PCR in the extracted total cellular RNA. The level of TH mRNA expression was standardized by level of RPL19 in each sample. C) Cells (1 × 10^5 viable cells) were cultured with the indicated concentrations of Aldo or Dex in the presence of 0.1 mM IBMX for 24 h, and the cAMP concentration in the culture medium was determined by enzyme immunoassay after acetylation of 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. untreated control in each group or between the indicated groups.

**Fig. 3. Involvement of genomic actions in TH upregulation by aldosterone in PC12 cells.** PC12 cells (3 × 10^5 viable cells) were precultured in DMEM containing 1% FCS
and 1% HS and cells were treated with aldosterone (Aldo; 100 nM) or dexamethasone (Dex; 1 µM) in combination with A) eplerenone, B) RU486, C) cycloheximide (CHX), D) and E) eplerenone plus RU486. After 24-h culture, the medium was removed, and total cellular RNAs were extracted. Steady-state mRNA levels of TH and RPL19 were analyzed by quantitative real-time RT-PCR in the extracted total cellular RNA. The level of TH mRNA expression was standardized by level of RPL19 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. untreated control in each group or between the indicated groups; n.s., not significant.

Fig. 4. Involvement of MAP kinases and other signaling pathways in TH upregulation by aldosterone in PC12 cells. After preculture, PC12 cells (3 × 10^5 viable cells) were treated with aldosterone (100 nM) or dexamethasone (1 µM) in combination with A) U0126, B) SB203580, C) SP600125, D) Y-27632, E) LY294002 and F) PKCI. After 24-h culture, the medium was removed, and total cellular RNAs were extracted. Steady-state mRNA levels of TH and RPL19 were analyzed by quantitative real-time RT-PCR in the extracted total cellular RNA. The level of TH mRNA expression was standardized by level of RPL19 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. untreated control in each group or between the indicated groups.
Fig. 5. Effects of eplerenone and CHX in combination with SAPK/JNK inhibition on aldosterone-induced TH expression and dopamine production in PC12 cells.

After preculture, PC12 cells ($3 \times 10^5$ viable cells) were treated with aldosterone (Aldo, 100 nM) with either combination of A) eplerenone and SP600125 or B) cycloheximide (CHX) and SP600125. After 24-h culture, the medium was removed, and total cellular RNAs were extracted. Steady-state mRNA levels of TH and RPL19 were analyzed by quantitative real-time RT-PCR in the extracted total cellular RNA. The level of TH mRNA expression was standardized by level of RPL19 in each sample. C) PC12 cells ($1 \times 10^5$ viable cells) were precultured in DMEM containing 1% FCS and 1% HS for 24 h, and then cells were treated with the indicated concentrations of Aldo in combination of eplerenone and SP600125. The culture medium was collected after 24-h culture and the catecholamine levels were determined by HPLC. 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. untreated control in each group or between the indicated groups.

Fig. 6. Effects of BMP-4 and BMP-7 on aldosterone- and dexamethasone-induced TH expression by PC12 cells. After preculture, PC12 cells ($3 \times 10^5$ viable cells) were treated with indicated concentrations of BMP-4 and -7 in the absence (A) or presence (B and C) of aldosterone (Aldo) or dexamethasone (Dex). After 24-h culture, the medium was removed, and total cellular RNAs were extracted. Steady-state mRNA levels of TH and RPL19 were analyzed by quantitative real-time RT-PCR in the extracted total cellular RNA. The level of TH mRNA expression was standardized by level of RPL19 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. untreated control in each group or between the indicated groups.

Fig. 7. Effects of BMP-4 and BMP-7 on aldosterone-induced dopamine production and MR expression and aldosterone effects on the BMP system in PC12 cells. A) PC12 cells (1 × 10^5 viable cells) were precultured in DMEM containing 1% FCS and 1% HS for 24 h, and then cells were treated with aldosterone (Aldo; 100 nM) and the indicated concentrations of BMP-4 or BMP-7. The culture medium was collected after 24-h culture and the catecholamine levels were determined by HPLC. Results are shown as mean ± SEM of data from at least three separate experiments, each performed with triplicate samples. *P < 0.05 between the indicated groups. B) After preculture, PC12 cells (3 × 10^5 viable cells) were treated with the indicated concentrations of BMP-4 and -7 for 24 h. Steady-state mRNA levels of MR and RPL19 were analyzed by quantitative real-time RT-PCR in the extracted total cellular RNA. The level of TH mRNA expression was standardized by level of RPL19 in each sample. C) After preculture, PC12 cells (1 × 10^5 viable cells) were treated with the indicated concentrations of BMP-4 and BMP-7 for 24 h. Cells were solubilized and the cell lysates were subjected to SDS-PAGE/immunoblotting (IB) analysis using anti-MR and anti-actin antibodies. K-562 whole cell lysate was used for a positive control of immunoblot analysis of MR detection. The results shown are representative of those obtained from three independent experiments. D) Cells were stimulated with BMP-4 (+BMP-4; 100 ng/ml) for 1 h in serum-free DMEM and immunofluorescence (IF) studies were performed with anti-phospho-Smad1,5,8 (pSmad1,5,8) antibody. Bars indicate 20 µm in size. For IB analysis, cells were incubated with Aldo (100 nM) or
Dex (1 µM) in combination with BMP-4 (100 ng/ml). After 60-min treatment, total cellular protein and the membrane fractions were extracted and subjected to IB analysis using anti-pSmad1,5,8 and anti-actin antibodies. The results shown are representative of those obtained from three independent experiments. E) Cells (3 × 10^5 viable cells) were precultured and treated with Aldo (10-100 nM) or dexamethasone (Dex; 0.1-1 µM). After 24-h culture, the medium was removed, and total cellular RNAs were extracted. Steady-state mRNA levels of BMP-4, BMP-7 and RPL19 were analyzed by quantitative real-time RT-PCR in the extracted total cellular RNA. The level of BMP mRNA expression was standardized by level of RPL19 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 vs. untreated control in each group.

Fig. 8. Effects of MR blockade on BMP-4 enhancement of aldosterone-induced catecholamine production by PC12 cells. A) After preculture, PC12 cells (3 × 10^5 viable cells) were treated with aldosterone (Aldo; 100 nM) in combination with the indicated concentrations of BMP-4 and eplerenone. After 24-h culture, the medium was removed, and total cellular RNAs were extracted. Steady-state mRNA levels of TH and RPL19 were analyzed by quantitative real-time RT-PCR in the extracted total cellular RNA. The level of TH mRNA expression was standardized by level of RPL19 in each sample. B) PC12 cells (1 × 10^5 viable cells) were precultured in DMEM containing 1% FCS and 1% HS for 24 h, and then cells were treated with Aldo (100 nM) in combination with the indicated concentrations of BMP-4 and eplerenone. The culture medium was collected after 24-h culture and the catecholamine levels were determined.
by HPLC. 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. untreated control in each group.

**Fig. 9. Effects of BMP-4 on aldosterone-induced activation of Rho-SAPK/JNK pathway in PC12 cells.** A) After preculture, cells were incubated with aldosterone (Aldo; 100 nM), BMP-4 (100 ng/ml) and SP600125 (10 µM) with either alone or indicated combinations. After adding Aldo for 10 min to 60 min, cells were lysed and subjected to SDS-PAGE/immunoblot (IB) analysis using anti-phospho-SAPK/JNK (pSAPK/JNK) and anti-total-SAPK/JNK (tSAPK/JNK) antibodies that detect phosphorylated MAPK signaling. The results shown are representative of those obtained from three independent experiments of 30-min exposure with Aldo. The relative integrated density of each protein band (p54- and p46-SAPK/JNK) was digitized by NIH image J 1.34s. 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. untreated control in each group or between the indicated groups. B) Cells were incubated with Aldo (100 nM) or dexamethasone (Dex, 1 µM) in combination with BMP-4 (100 ng/ml). After adding aldosterone for 60 min, total cellular protein and the membrane fractions were extracted and then subjected to IB analysis using anti-RhoA antibody. The results shown are representative of those obtained from three independent experiments. The relative integrated density of each protein band was digitized by NIH image J 1.34s. 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. untreated control or between the indicated groups.

**Fig. 10. Inhibitory effects of Rho-SAPK/JNK signaling on BMP-4 enhancement of aldosterone-induced TH expression in PC12 cells.** After preculture, PC12 cells (3 × 10^5 viable cells) were treated with aldosterone (Aldo; 100 nM) and BMP-4 (100 ng/ml) in combination with SP600125, *Clostridium difficile* Toxin B or GGTI-298. After 24-h culture, the medium was removed, and total cellular RNAs were extracted. Steady-state mRNA levels of TH and RPL19 were analyzed by quantitative real-time RT-PCR in the extracted total cellular RNA. The level of TH mRNA expression was standardized by level of RPL19 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. untreated control in each group or between the indicated groups.

**Fig. 11. A possible mechanism of BMP-4 and aldosterone-induced catecholamine production in the adrenomedullar cells.** Aldosterone stimulated dopamine production and TH mRNA expression through genomic and nongenomic actions including MR-dependent and independent SAPK/JNK signaling. BMP-4 enhanced aldosterone-induced dopamine production and TH mRNA expression via MR by upregulating nongenomic actions through Rho-SAPK/JNK pathways.
New Fig. 1

A

Morphological changes of PC12 cells

B

IB: MR
IB: Actin

C

D

IHC: anti-BMP-4 antibody
New Fig. 3

A

B

C

D

E

TH mRNA levels (folds)

TH mRNA levels (folds)

TH mRNA levels (folds)

TH mRNA levels (folds)

TH mRNA levels (folds)

+ Aldo (100 nM)  
+ Dex (1 μM)

+ RU486 (nM)

+ Eplerenone (nM)

+ CHX (μg/ml)

Aldo (nM)  
Eple (nM)  
RU486 (nM)  
Dex (μM)  
Eple (nM)  
RU486 (nM)

0  
0  
0  
1  
0  
0

100  
100  
100  
300  
300  
300

300  
300  
300  
1  
1  
1

1000  
1000  
1000  
1  
100  
100

0  
0  
0  
n.s.  
n.s.  
n.s.

0  
0  
0  
n.s.  
n.s.  
n.s.
New Fig. 5
New Fig. 6

A

B

C

TH mRNA levels (folds)

Control 30 100 30 100

+ BMP-4 (ng/ml) + BMP-7 (ng/ml)

TH mRNA levels (folds)

Aldo (nM) 0 100 100 100 0 0 0 0

Dex (μM) 0 0 0 0 0 1 1 1

BMP-4 (ng/ml) 0 0 30 100 0 0 30 100

TH mRNA levels (folds)

Aldo (nM) 0 100 100 100 0 0 0 0

Dex (μM) 0 0 0 0 0 1 1 1

BMP-7 (ng/ml) 0 0 30 100 0 0 30 100
Fig. 8

A

TH mRNA levels (folds)

Aldo (nM) 0 100 100 100 100 100 100
Eplerenone (nM) 0 0 0 100 100 300 300
BMP-4 (ng/ml) 0 0 100 0 100 0 100

B

Dopamine levels (folds)

Aldo (nM) 0 100 100 100 100 100 100
Eplerenone (nM) 0 0 0 100 100 300 300
BMP-4 (ng/ml) 0 0 100 0 100 0 100
New Fig. 9

A

IB: pSAPK/JNK
IB: I SAPK/JNK

Relative density of pSAPK/JNK (top)

Relative density of pSAPK/JNK (bottom)

Aldo (nM) 0 0 100 0 100 100 100
BMP-4 (ng/ml) 0 0 0 0 0 0 100
SP600125 (μM) 0 0 0 0 0 0 10

B

IB: membrane Rho A
IB: total Rho A

Relative density of membrane Rho A (top)

Relative density of membrane Rho A (bottom)

Aldo (nM) 0 0 100 0 100 100
Dex (μM) 0 0 0 0 0 0
BMP-4 (ng/ml) 0 0 0 0 0 100
New Fig. 10
Fig. 11

Aldosterone

MR

BMP-4 +

Rho

SAPK/JNK

Nongenomic action

Genomic action

Stimulation of TH expression

catecholamine production