Antagonistic effects of bone morphogenetic protein-4 and -7 on renal mesangial cell proliferation induced by aldosterone through MAPK activation

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Submitted 11 October 2006; accepted in final form 21 January 2007

Otani H, Otsuka F, Inagaki K, Takeda M, Miyoshi T, Suzuki J, Mukai T, Ogura T, Makino H. 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–F1525, 2007. First published January 23, 2007; doi:10.1152/ajprenal.00402.2006.—Aldosterone and angiotensin II (ANG II) contribute to the development and progression of renal damage. Here we investigated the effects of bone morphogenetic proteins (BMPs) on renal cell proliferation evoked by aldosterone and ANG II with mouse mesangial cells, which express mineralocorticoid receptors (MR), ANG II type 1 receptors, and BMP signaling molecules. Aldosterone and ANG II stimulated mesangial cell mitosis and activated ERK1/2 and SAPK/JNK signaling. These aldosterone effects were neutralized by the MR antagonist eplerenone and inhibition of transcription or translation, suggesting the involvement of genomic activation via MR. BMP-4 and BMP-7 stimulated Smad1, -5, -8 signaling more potently than BMP-2 and BMP-6, leading to suppression of mesangial cell mitosis and MR expression. MAPK inhibitors including U-0126 and SP-600125, but not SB-203580, suppressed aldosterone-induced cellular DNA synthesis, implying that ERK1/2 and SAPK/JNK pathways play crucial roles in mesangial cell proliferation. BMP-4 and BMP-7 inhibited phosphorylation of ERK1/2 and SAPK/JNK induced by aldosterone while activating p38 pathway, resulting in inhibition of aldosterone-induced cell mitosis. In contrast, aldosterone modulated the mesangial BMP system by decreasing expression of ALK-3, BMP-4, and BMP-7 while increasing inhibitory Smad6 expression. Thus novel functional cross talk between the mesangial BMP system and aldosterone signaling was uncovered, in which inhibition of MAPK signaling and MR expression by BMP-4 and BMP-7 may be involved in ameliorating renal damage due to mesangial proliferation caused by aldosterone.

angiotensin II; kidney; mineralocorticoid

THERE IS INCREASING EVIDENCE suggesting that aldosterone exerts important physiologically and/or pathophysiologically relevant effects on the cardiovascular system, kidney, and brain in addition to its well-established role in regulating body electrolyte and water homeostasis. Recent studies have shown that aldosterone directly induces renal glomerular damage by increasing extracellular matrix as well as stimulating mesangial cell proliferation as demonstrated in remnant nephropathy model. In addition, experiments using Wistar-Furth rats, an inbred strain that is resistant to mineralocorticoids (7, 62), demonstrated that renal damage on 5/6 nephrectomy was markedly reduced independent of any reduction in blood pressure (14). In contrast, remnant renal glomeruli of control Wistar rats showed mesangial proliferation, mesangial fibrosis, and crescent formation. These results have placed aldosterone as a potentially important component of the renin-angiotensin-aldosterone system in this remnant nephropathy model.

Renal histological injuries have been observed in patients with primary aldosteronism (10, 16), including characterization of glomerulosclerosis, tubular atrophy, interstitial fibrosis, and arteriosclerosis. Detailed pathological studies revealed that glomerulosclerotic and arteriosclerotic changes were significantly elevated in patients with residual hypertension compared with those with normotension after adrenalectomy (22). Physiological studies revealed that patients with primary aldosteronism display glomerular hyperfiltration (58), which is often unmasked after suppression of excess aldosterone by surgery or spironolactone administration. More recent reports (60, 63) also concluded that glomerular filtration rate and albuminuria are higher in these patients compared with essential hypertension. Thus hypertension caused by chronic excess aldosterone secretion is characterized by earlier and more prominent renal damage than essential hypertension (60). Renal dysfunction in hyperaldosteronism is possibly related to functional adaptation of the kidney to the actions of excess aldosterone (63). However, considering the experimental evidence linking aldosterone to renal damage (26), aldosterone may directly accelerate substantial glomerular damages in the presence of prolonged hypertension caused by primary aldosteronism.

Mesangial proliferation is a key component of glomerular damages that are attributed to the effects of aldosterone. In vivo studies by Nishiyama et al. (49) demonstrated that administration of aldosterone and salt to Sprague-Dawley rats re-
sulted in glomerular injury characterized by mesangial matrix expansion and mesangial cell proliferation with increased proliferating cell nuclear antigen-positive cells. This glomerular damage was significantly suppressed by concurrent administration of an aldosterone antagonist, eplerenone. Gullu et al. (19) also reported that aldosterone blockade by spironolactone ameliorates not only glomerular fibrosis but also mesangial cell proliferation shown in mesangioproliferative glomerulonephritis produced by anti-thy1.1 nephritis rats. These findings suggest that aldosterone is directly involved in mesangial proliferation in vivo. Activation of the mitogen-activated protein kinase (MAPK) pathway in renal cortical tissue (49) and mesangial cells (48) via mineralocorticoid receptor (MR) has been proposed as the mechanism underlying the aldosterone-induced mesangial cell proliferation. In the present study, we focused on mesangial cell proliferation facilitated by excess aldosterone through MAPK activation.

Bone morphogenetic proteins (BMPs), which belong to the transforming growth factor (TGF)-β superfamily, have been widely recognized as important molecules involved in renal differentiation (65). Recent studies have provided the evidence that BMPs, and in particular BMP-7, play a key role in the pathogenesis of various renal diseases including early experimental diabetic (79) or obstructive (24) nephropathy. BMPs were originally identified as the active components in bone extracts capable of inducing bone formation at ectopic sites. Recently, a variety of physiological BMP actions in many endocrine tissues including the ovary (54, 64), pituitary (53), thyroid (68), and adrenals (27, 31, 67) have been clarified.

Recently, a variety of physiological BMP actions in many endocrine tissues including the ovary (54, 64), pituitary (53), thyroid (68), and adrenals (27, 31, 67) have been clarified. Among BMP ligands, BMP-7 plays pivotal roles during embryonic renal and eye development (12); however, BMP-7 expression is retained in only a few tissues in adults, most prominently in the kidney (34). Nevertheless, its physiological functions in the adult kidney are still unknown.

In the present study, we examined aldosterone and ANG II effects on mesangial cell proliferation with a mouse mesangial cell line. We also investigated the antagonistic effects of BMPs that have been recently shown to ameliorate various renal and vascular damage (65).

**MATERIALS AND METHODS**

**Reagents and supplies.** A 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium (DMEM-F-12), penicillin-streptomycin solution, ANG II acetate salt, d-aldosterone, actinomycin D (ACD), cycloheximide (CHX), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant human TGF-β1 and PDGF-BB were obtained from PeproTech EC (London, UK). Recombinant human BMP-2, -4, -6, and -7 and actin A were purchased from R&D Systems (Minneapolis, MN), and U-0126 and SB-203580 were from Promega (Madison, WI). SP-600125 was from Biomol Labs (Plymouth Meeting, PA). Eplerenone and candesartan (CV-11003) were provided by Pfizer (New York, NY) and Takeda Chemical Industries (Osaka, Japan), respectively. Plasmids of Id-1-Luc were kindly provided by Drs. Tetsuro Watabe and Kohei Miyazono (Tokyo University, Tokyo, Japan).

**Mesangial cell culture.** The mouse glomerular mesangial cell line SV40 MES13 (40) was from American Type Culture Collection (Manassas, VA). MES13 cells were cultured in DMEM-F-12 supplemented with 5% fetal calf serum (FCS) and penicillin-streptomycin solution at 37°C under a humid atmosphere of 95% air-5% CO2. Cell numbers were counted by culturing MES13 cells in 24-well plates (1 × 105 viable cells) with serum-free DMEM-F-12 for 24 h. The cells were then washed with PBS, trypsinized, and applied to a Coulter counter (Beckman Coulter, Fullerton, CA).

**RNA extraction, RT-PCR, and quantitative real-time PCR analysis.** To prepare total cellular RNA, MES13 cells were cultured in 12-well plates (5 × 105 viable cells) and treated with indicated concentrations of ANG II, aldosterone, or growth factors including BMPs in serum-free DMEM-F-12. After 24-h culture, the medium was removed, and total cellular RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA), quantified by measuring absorbance at 260 nm, and stored at −80°C until assay. Total RNA from adult male BALB/c mouse kidneys was also extracted with TRIzol. The expression of BMP ligands, BMP receptors, follistatin, Smads, ANG II type 1 receptor (AT1R), and MR mRNAs were detected by RT-PCR analysis. The extracted RNA (1.0 µg) was subjected to an RT reaction with the First-Strand cDNA synthesis system (Invitrogen) with random hexamer (2 ng/µl), reverse transcriptase (200 U), and deoxynucleotide triphosphate (0.5 mM) at 42°C for 50 min and 70°C for 10 min. Subsequently, hot-start PCR was performed with MgCl2 (1.5 mM), deoxynucleotide triphosphate (0.2 mM), and 2.5 U of Taq DNA polymerase (Invitrogen) under the conditions we previously reported (70). Oligonucleotides used for RT-PCR were custom-ordered from Kurabo Biomedical (Osaka, Japan). PCR primer pairs were selected from different exons of the corresponding genes as follows: activin receptor-like kinase (ALK)-2, 639–661 and 1100–1121 (from GenBank accession no. NM_007394); ALK-3, 426–445 and 916–935 (from NM_009758); ALK-4, 130–150 and 637–657 (from NM_007395); ALK-6, 598–617 and 1034–1053 (from NM_007560); activin type II receptor (ActRII), 611–630 and 1083–1102 (from NM_037396); BMP type II receptor (BMPRII), 13–32 and 515–534 (from NM_007561); Smad1, 878–899 and 1193–1214 (from NM_008539); Smad2, 111–131 and 353–555 (from NM_010754); Smad3, 474–494 and 828–848 (from NM_016769); Smad4, 712–732 and 1082–1102 (from NM_008540); Smad5, 636–658 and 1062–1083 (from NM_008541); Smad6, 1081–1101 and 1527–1547 (from NM_008542); Smad7, 1912–1932 and 2328–2348 (from AA000550); Smad8, 271–291 and 538–558 (from AF175408); follistatin, 361–385 and 525–550 (from NM_008046); MR, 2978–2999 and 3219–3240 (from M36074); AT1R, 210–232 and 471–492 (from NM_177322); BMP-4, 220–240 and 430–440 (from NM_007754); BMP-7, 415–438 and 684–704 (from X56906); and a housekeeping gene, ribosomal protein L19 (RPL19), 373–393 and 547–567 (from NM_009078). Aliquots of PCR products were electrophoresed on 1.5% agarose gels and visualized after ethidium bromide staining. For the quantification of ALK-3, ActRII, BMPRII, Smad6, BMP-4, BMP-7, MR, and AT1R mRNA levels, real-time PCR was performed with the LightCycler-FastStart DNA Master SYBR Green I system (Roche Diagnostic, Tokyo, Japan) under conditions of annealing at 60–62°C with 4 mM MgCl2, following the manufacturer’s protocol. Accumulated levels of fluorescence were analyzed by the second derivative method after melting curve analysis (Roche Diagnostic), and then the expression levels of target genes were standardized by RPL19 level in each sample.

**Thymidine incorporation assay.** Cells (5 × 10³ viable cells) were precultured in 24-well plates with DMEM-F-12 containing 5% FCS for 24 h. After preculture medium was replaced with fresh serum-free medium and indicated combinations of ANG II, aldosterone, eplerenone, eplerenone, and BMPs, cell viability was determined by the MTS assay. Subsequently, cells were then washed with phosphate-buffered saline (PBS), incubated with 10% ice-cold trichloroacetic acid for 60 min at 4°C, and solubilized in 0.5 M NaOH, and radioactivity was determined with a liquid scintillation counter (TRI-CARB 2300TR, Packard, Meriden, CT).

**Western immunoblot analysis.** Cells (1 × 10⁵ viable cells) were precultured in 12-well plates in DMEM-F-12 containing 5% FCS for
24 h. After preculture, medium was replaced with serum-free fresh medium, and then indicated concentrations of ANG II, aldosterone, eplerenone, MAPK inhibitors, and growth factors including BMPs were added to the culture medium. After stimulation with hormones or growth factors for indicated periods (15 min to 48 h), cells were solubilized in 100 μl of RIPA lysis buffer (Upstate Biotechnology, Lake Placid, NY) containing 1 mM Na3VO4, 1 mM sodium fluoride, 2% SDS, and 4% 3-mercaptoethanol. The cell lysates were then subjected to SDS-PAGE and immunoblotting analysis as we previously reported (27, 68), with anti-phospho-Smad1, -5, -8 antibody (Cell Signaling Technology, Beverly, MA), anti-phospho- and anti-total extracellular signal-regulated kinase (ERK)1/2 MAPK antibody (Cell Signaling Technology), anti-phospho- and anti-total-p38 MAPK antibody (Cell Signaling Technology), anti-phospho- and anti-total-stress-activated protein kinase (SAPK)/c-Jun NH2-terminal kinase (JNK) MAPK antibody (Cell Signaling Technology), anti-MR antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-actin antibody (Sigma-Aldrich). For immunoblot analysis of MR detection, a positive control from K-562 whole cell lysate (Santa Cruz Biotechnology) was utilized.

Immunofluorescence microscopy. MES13 cells were precultured in serum-free DMEM-F-12, and then cells at ~50% confluence were treated with BMP-4 and -7 (100 ng/ml). After 1-h stimulation, cells were fixed with 4% formaldehyde in PBS, permeabilized with 0.5% Triton X-100 in PBS at room temperature, and washed three times with PBS. The cells were then incubated with anti-phospho-Smad1, -5, -8 antibody for 1 h and washed three times with PBS. Cells were then incubated with Alexa Fluor 488 anti-rabbit IgG (Invitrogen) in humidified chamber for 1 h and washed with PBS, and then stained cells were visualized under a fluorescent microscope.

Transient transfection and luciferase assay. MES13 cells (3 × 10^4 viable cells) were precultured in 12-well plates in DMEM-F-12 with 5% FCS for 24 h. The cells were then transiently transfected with 500 ng of Id-1-Luc reporter plasmid and 50 ng of cytomegalovirus-β-galactosidase plasmid (pCMV-β-gal) with FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN) for 24 h. The cells were then treated with BMP-2, -4, -6, and -7 and TGF-β1 (100 ng/ml) in fresh serum-free medium for 6, 12, and 24 h. The cells were washed with PBS and lysed with Cell Culture Lysis Reagent (Toyobo, Osaka, Japan). Luciferase activity and β-gal activity of the cell lysate were measured by luminescencer-PSN (ATTO, Tokyo, Japan) as we previously reported (44). The data are shown as the ratio of luciferase to β-gal activity.

cDNA array analysis. The GEArray system (SuperArray Bioscience, Frederick, MD), which includes 96 genes of mouse TGF-β and BMP signaling pathway, was used for analyzing the expression pattern of the BMP signaling system in MES13 cells. As we previously reported (44), extracted total RNAs (2.0 μg) were used as templates to generate biotin-16-dUTP-labeled cDNA probes according to the manufacturer’s instructions. The cDNA probes were denaturated and hybridized at 60°C with the cDNA array membranes, which were washed and exposed to X-ray films with the use of chemiluminescent substrates. To analyze the array results, we scanned the X-ray film and the image was inverted as grayscale TIFF files. The spots were digitized and analyzed with GEArray analyzer software (SuperArray Bioscience), and the data were normalized by subtraction of the background as the average intensity levels of three spots containing plasmid DNA of pUC18. The averages of two spots of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and four spots of cyclophilin A (PPA) were used as positive controls to compare the membranes. Using these standardized data, we compared the signal intensity of the membranes with the GEArray analyzer program (SuperArray Bioscience).

Statistical analysis. All results are shown as means ± SE of data from at least three separate experiments, each performed with triplicate samples. Differences between groups were analyzed for statistical significance with ANOVA (StatView 5.0 software, Abacus Concepts, Berkeley, CA). P values <0.05 were accepted as statistically significant.

RESULTS

First, we examined the mRNA expression of AT1R and MR in MES13 cells and mouse control kidney tissue by RT-PCR. In MES13 cells, AT1R (AT1A7R) and MR mRNA was clearly expressed as shown in mouse kidney tissues (Fig. 1). BMP ligands BMP-4 and BMP-7, BMP type I receptors ALK-2 (also called ActRIA), ALK-3 (BMPRIA), and ALK-4 (ActRIB), BMP type II receptors ActRII and BMPRII, and the binding protein follistatin were expressed in this cell line and in whole mouse kidneys. In addition, mRNA expression of Smad signaling molecules including Smad1, -2, -3, -4, -5, and -8 and inhibitory Smad6, -7 was also detected (Fig. 1). No detectable ALK-6 (BMPRIB) mRNA was observed in the MES13 cells.

As shown in Fig. 2A, ANG II and aldosterone stimulated thymidine incorporation of MES13 cells in a concentration-dependent manner, showing saturating effects of ANG II and aldosterone at 30 and 100 nM, respectively. Importantly, the mitotic effects elicited by aldosterone were neutralized by treatment with a specific MR antagonist, eplerenone.

Statistical analysis was performed using ANOVA (StatView 5.0 software, Abacus Concepts). P values <0.05 were accepted as statistically significant.
effects induced by ANG II were not significantly changed by eplerenone but, however, were blocked by an AT1R antagonist, candesartan (Fig. 2B). Likewise, mesangial cell number after treatment with ANG II and aldosterone showed concentration-dependent increases (Fig. 2C), in which eplerenone and candesartan suppressed mesangial cell proliferation induced by aldosterone and ANG II, respectively. These data suggest that the effects of ANG II and aldosterone on mesangial cell mitosis are mediated through AT1R and MR, respectively.

Next we investigated the molecular signaling mechanisms of aldosterone in MES13 cells. As shown in Fig. 3, A and B, aldosterone induced ERK1/2, SAPK/JNK, and, less potently, p38 phosphorylation. ANG II induced ERK1/2 and SAPK/JNK but not p38 phosphorylation. Eplerenone treatment abolished aldosterone-induced ERK1/2 and SAPK/JNK phosphorylation but did not affect ANG II-induced activation of ERK1/2 and SAPK/JNK (Fig. 3, A and B). Thus eplerenone abolished aldosterone-induced ERK1/2 and SAPK/JNK phosphorylation in parallel with its action on aldosterone-induced cell mitosis. Aldosterone-induced ERK1/2 phosphorylation at 15 min and 1 h was impaired by pretreatment with a transcriptional inhibitor, ACD (5 μg/ml) and a translational inhibitor, CHX (5 μg/ml) (Fig. 3C), suggesting the involvement of genomic actions by aldosterone via MR in mesangial cell mitosis.

As shown in Fig. 4, the presence of BMP signaling was determined by Western immunoblotting using anti-phospho-Smad1, -5, -8 antibody. BMP-4 and BMP-7 efficaciously stimulated Smad1, -5, -8 phosphorylation compared with BMP-2 and BMP-6 for a 6-h observation. As expected, TGF-β1 did not stimulate Smad1, -5, -8 phosphorylation, demonstrating specificity of Smad signaling in this cell line. Furthermore, the immunofluorescence study clearly demonstrated nuclear localization of the phosphorylated Smad1, -5, and -8 molecules by treatments with BMP-4 and BMP-7. The activation of Smad1, -5, -8 signaling was further quantified by luciferase analysis using the promoter activity of a BMP target gene, Id-1 (Fig. 4B). Collectively, these studies revealed that BMP-4 and BMP-7 were the most potent BMP ligands for induction of Smad1, -5, and -8 signaling in MES13 cells.

To characterize the functional relevance of this mesangial BMP system, effects of BMP ligands on cell mitosis were tested. In accordance with the results shown in Smad1, -5, and -8 activation (Fig. 4), BMP-4 and BMP-7 elicited inhibitory effects on MES13 cell mitosis among four BMP ligands in a concentration-dependent manner (Fig. 5A). It is noteworthy that ANG II- and aldosterone-induced mitotic activity was abrogated by treatment with BMP-4 and BMP-7, although the effects of BMP-2 and BMP-6 on mitotic inhibition were marginal in mesangial cells (Fig. 5B). As shown in Fig. 5C, cell counting experiments also confirmed the inhibitory effects of BMP-4 and BMP-7 on mesangial cell proliferation induced by aldosterone.

As demonstrated in Fig. 6, the detectable levels of phosphorylated ERK1/2, p38, and SAPK/JNK were observed 15 min after treatment with aldosterone and reached maximum activation levels at 1 h. At each time point observed for 12 h, treatments with BMP-4 and BMP-7 inhibited aldosterone-induced phosphorylation of ERK1/2 and SAPK/JNK. The effects at the maximal phase (1-h treatment) were quantified by densitometric analysis (Fig. 7). This shows that ANG II and aldosterone preferentially activated ERK1/2 and SAPK/JNK

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**Fig. 2.** Effects of ANG II, aldosterone, and eplerenone on mitotic action by mouse mesangial cells. Cells (5 × 10^3 viable cells) were treated with indicated concentrations (nM) of ANG II and aldosterone (Aldo) in the absence (A) or presence (B) of eplerenone (Ep) and candesartan (CV) for 24 h, and thymidine uptake assay was performed. For counting cell number (C), cells (1 × 10^5 viable cells) were cultured in serum-free medium with indicated concentrations of ANG II and Aldo in combination with Ep and CV for 24 h. Results are shown as means ± SE of data from at least 3 separate experiments, each performed with triplicate samples. *P < 0.05, **P < 0.01 vs. control and between the indicated groups.
signaling while BMP-4 and BMP-7 significantly suppressed ERK1/2 and SAPK/JNK activation induced by ANG II and aldosterone. BMP-4 and BMP-7 induced p38 phosphorylation more potently than aldosterone. Interestingly, the phosphorylation of Smad1, -5, and -8 induced by BMP-4 and BMP-7 was significantly impaired by aldosterone, while it was not affected by ANG II (Fig. 7B).

To examine whether MAPK is actually involved in mesangial cell mitosis, cells were treated with specific MAPK inhibitors. U-0126, SB-203580, and SP-600125 inhibited ERK1/2, p38, and SAPK/JNK phosphorylation induced by aldosterone, respectively (Fig. 8A). As seen in Fig. 8B, treatments with U-0126 showed a potent reduction of cellular DNA synthesis compared with corresponding concentrations of DMSO solvent, SB-203580, and SP-600125 in a concentration-dependent manner. Notably, aldosterone-induced thymidine uptake was suppressed by 3 μM U-0126 and SP-600125 but not by SB-203580 (Fig. 8C), suggesting that ERK1/2 and SAPK/JNK activation is critically involved in the cellular mitosis stimulated by aldosterone.

Since these aldosterone effects appeared to occur through MR, we examined changes of MR expression levels in MES13 cells. Quantitative real-time PCR analysis showed that ANG II (30 nM) reduced AT1R mRNA levels while aldosterone (100 nM) reduced MR expression in this cell line (Fig. 9A), suggesting the presence of a functional feedback system in ANG II-aldosterone regulation in MES13 cells. Among the ligands tested, BMP-4 and BMP-7 specifically decreased MR mRNA expression of mesangial cells as shown in the cells treated with aldosterone (Fig. 9A). The expression of MR protein was examined by Western immunoblot analysis. The specific bands for MR protein (~102 kDa) were confirmed in total cell lysates, and BMP-4 and BMP-7 decreased the levels of mesangial MR protein in a time-dependent manner, while the levels of an internal control actin protein were unchanged (Fig. 9B). These data suggest that BMP-4 and BMP-7 suppress aldosterone-induced cell proliferation, at least in part, through inhibiting MR expression in mesangial cells.

Aldosterone effects on the mesangial BMP system were examined with a cDNA array that includes genes for mouse TGF-β and BMP signaling pathway. Based on the quantitative analysis of the arrays (Fig. 10A), it was of note that aldosterone and ANG II showed differential pattern of key BMP signaling molecules including ALK-3, ActRII, BMPRII, and Smad6. These cDNA array data were further confirmed by quantitative real-time PCR analysis, demonstrating that aldosterone de-
creased ALK-3 (BMPRIA) expression while ANG II upregulated ActRII and BMPRII (Fig. 10B). Interestingly, ANG II as well as aldosterone potently increased the expression of inhibitory Smad6, suggesting the existence of a functional link between circulating ANG II-aldosterone and the mesangial BMP system. The changes of BMP receptors and Smad expression by aldosterone were reflected in modulation of BMP-induced Smad1, -5, and -8 phosphorylation (see Fig. 7B). In addition, the expression of BMP-4 and BMP-7 was significantly decreased by treatment with ANG II and aldosterone in mesangial cells (Fig. 10B). The reduction of endogenous BMP-4 and BMP-7 may also be involved in the enhancement

Fig. 4. Effects of BMP ligands on Smad1, -5, and -8 activation in mouse mesangial cells. A: cells (1 × 10⁵ viable cells) were precultured for 24 h and stimulated with BMP-2, -4, -6, and -7 and transforming growth factor (TGF)-β1 (100 ng/ml). After 1-, 3-, and 6-h culture, cells were lysed and subjected to SDS-PAGE and immunoblotting (IB) analysis using anti-phospho-Smad1, -5, -8 (pSmad1,5,8) antibody. Immunofluorescence (IF) studies were also performed with pSmad1,5,8 antibody on BMP-treated cells for 1 h. B: cells (3 × 10⁵ viable cells) were transiently transfected with Id-1-Luc reporter plasmid (500 ng) and pCMV-β-galactosidase (β-gal). After treatment with BMP-2, -4, -6, and -7 and TGF-β1 (100 ng/ml) for 6, 12, and 24 h, cells were lysed and the luciferase activity was measured. Data were analyzed as the ratio of luciferase to β-gal activity. Results are shown as means ± SE of data from at least 3 separate experiments, each performed with triplicate samples. *P < 0.05, **P < 0.01 vs. corresponding time control; #P < 0.01 vs. BMP-2 group.

Fig. 5. Effects of BMPs on ANG II- and aldosterone-induced mesangial cell mitosis. Cells (5 × 10⁵ viable cells) were treated with BMP-2, -4, -6, and -7 and TGF-β1 (A) or ANG II (30 nM) and Aldo (100 nM) in combination with BMPs (100 ng/ml) (B) for 24 h, and thymidine uptake assay was performed. For counting cell number (C), cells (1 × 10⁴ viable cells) were cultured in serum-free medium with Aldo in combination with BMPs for 24 h. Results are shown as means ± SE of data from at least 3 separate experiments, each performed with triplicate samples. *P < 0.05, **P < 0.01 vs. each control or between the indicated groups.
animal hypertensive models. In animal hypertensive models, MR antagonism elicits renal protection independently of antihypertensive mechanisms (6). The mechanism whereby aldosterone induces renal injury is related to the induction of reactive oxygen (49) and increased expression of inflammatory cytokines (4). However, the pathways involved in the progres-

DISCUSSION

The kidney exhibits a high density of MR expression pattern along the tubular epithelium of the distal tubules and collecting ducts (72). MR is also detected at glomeruli (72) and preglomerular vasculature (73) in the kidney. Aldosterone not only governs transport of water and electrolytes by acting as the principal ligand for MR but also causes renal injury in various

Fig. 6. Time course effects of MAPK phosphorylation in mouse mesangial cells. Cells (1 x 10^5 viable cells) were precultured for 24 h and stimulated with ANG II (30 nM) and Aldo (100 nM) in the absence or presence of BMP-4 and BMP-7 (100 ng/ml). After 15-min (m), and 1-, 3-, 6-, and 12-h culture, cells were lysed and subjected to SDS-PAGE and IB analysis using anti-pERK1/2, anti-pp38, and anti-pSAPK/JNK antibodies. Results shown are representative of those obtained from 3 independent experiments.

Fig. 7. Effects of BMPs on MAPK activation induced by ANG II and Aldo. A: cells (1 x 10^5 viable cells) were stimulated with ANG II (30 nM) and Aldo (100 nM) in the absence or presence of BMP-4 and BMP-7 (100 ng/ml). After 1-h culture, cells were lysed and subjected to SDS-PAGE and IB analysis using anti-pSmad1,5,8, anti-pERK1/2, anti-tERK1/2, anti-pp38, anti-tp38, anti-pSAPK/JNK, and anti-tSAPK/JNK antibodies. B: relative integrated density of each protein band was digitized by NIH ImageJ 1.34s. Results are shown as means ± SE of data from at least 3 separate experiments, each performed with triplicate samples. *P < 0.05, **P < 0.01 vs. control or between the indicated groups.
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sion from inflammation to glomerular injury and tubulointerstitial fibrosis remain uncertain.

In the present study, a novel functional interrelationship between aldosterone actions and BMPs was uncovered in renal mesangial cells. Several preferential combinations of BMP ligands and receptors have been recognized to date, e.g., BMP-2 and BMP-4 and growth and differentiation factor (GDF)-5 preferentially bind to ALK-3 and/or ALK-6, BMP-6 and BMP-7 most readily bind to ALK-2 and/or ALK-6 (2, 13, 71, 82), and BMP-15 efficiently binds to ALK-6, with much lower affinity for ALK-3 (45). Regarding type II receptors, ActRII, which was originally identified as an activin receptor, also acts as receptor for BMP-6 and BMP-7 (13, 82), while BMPRII binds exclusively to BMP ligands including BMP-2, -4, -6, -7, and -15 (38, 45, 50, 59). Among the BMP ligands tested, our present data showed that BMP-4 and BMP-7 exerted effective activation of Smad signaling and mitotic inhibition of mesangial cells compared with BMP-2 and BMP-6. Since ALK-6 is not expressed in MES13 cells, receptor pairs of ALK-3 and BMPRII and those of ALK-2 and ActRII/BMPRII are likely to be the major functional complex for BMP-4 and BMP-7, respectively.

Thus far, it appears that BMP-4 and BMP-7 have more important roles in renal development than other members of the BMP family, each of which has unique effects on different parts of the developing kidney (42). BMP-7 prevents metanephric mesenchymal cells from undergoing apoptosis and maintains them in an arrested development-type situation (74), while promoting expansion of the stromal progenitor cell population in the presence of fibroblast growth factor-2 (11). The roles of BMP-4 in the developing kidney appear to be different based on the studies using targeted mutation and cell culture. BMP-4 inhibits the ureteric budding process but promotes the growth or elongation of the stalk of the ureteric bud, showing a unique function in kidney branching morphogenesis (35, 57).

In the present study, aldosterone-induced cell mitosis was abolished by treatment with BMP-4 and BMP-7 through the suppression of ERK1/2 and SAPK/JNK signaling. The finding that chemical inhibition of ERK1/2 and SAPK/JNK restored mitotic activity stimulated by aldosterone supports the concept that activation of these MAPK pathways leads to cellular mitogenesis due to aldosterone. Another MAPK pathway, p38 signaling, which has been suggested as an apoptotic pathway in the kidney (37, 51, 55), was activated by aldosterone but not by ANG II. Aldosterone effects on activating p38 pathway have been reported in rat vascular smooth muscle cells (VSMCs) (8, 9), in which the p38 pathway is likely to play a key role in profibrotic and proinflammatory actions induced by aldosterone through the Src-dependent pathway. Since blockage of the p38 pathway by SB-203580 had no significant effect on aldosterone-induced cell mitosis, the role of p38 in aldosterone-induced cell mitosis appears to be minimal.

Fig. 8. Effects of MAPK inhibitors on mesangial cell mitosis induced by Aldo. A: cells (1 x 10^5 viable cells) were precultured for 24 h and then stimulated with Aldo in the absence or presence of U-0126, SB-203580, and SP-600125. After 1-h culture, cells were lysed and subjected to SDS-PAGE and IB analysis using anti-pERK1/2, anti-tERK1/2, anti-p38, anti-t38, anti-pSAPK/JNK, and anti-tSAPK/JNK antibodies. B: cells (5 x 10^5 viable cells) were treated with DMSO alone, U-0126, SB-203580, or SP-600125 (0.3–10 μM; each containing 0.003–0.1% DMSO) for 24 h, and thymidine uptake assay was performed. Results are shown as means ± SE of data from at least 3 separate experiments, each performed with triplicate samples. *P < 0.05, **P < 0.01 vs. DMSO control. C: cells (5 x 10^5 viable cells) were treated with U-0126, SB-203580, or SP-600125 (3 μM; each containing 0.03% DMSO) in the presence or absence of Aldo for 24 h, and thymidine uptake assay was performed. Results are shown as means ± SE of data from at least 3 separate experiments, each performed with triplicate samples. **P < 0.01 vs. control group or between the indicated groups. AJP-Renal Physiol • VOL 292 • MAY 2007 • www.ajprenal.org
Fig. 9. Effects of BMPs on MR expression in mouse mesangial cells. A: after preculture, the cells were treated with ANG II (30 nM), Aldo (100 nM), BMP-2, -4, -6, -7, activin A, or PDGF-BB (100 ng/ml) for 24 h. Total cellular RNA was extracted, and MR and AT1R mRNA levels were examined by real-time RT-PCR. The expression levels of target genes were standardized by ribosomal protein L19 (RPL19) level in each sample. Results are shown as means ± SE of data from at least 3 separate experiments, each performed with triplicate samples. *P < 0.05, **P < 0.01 vs. control.

B: cells (1 x 10⁵ viable cells) were precultured for 24 h and stimulated with BMP-4 and BMP-7 (100 ng/ml). After 6-, 12-, 24- and 48-h culture, cells were lysed and subjected to SDS-PAGE and IB analysis using anti-MR antibody and anti-actin antibody. Arrowhead indicates a MR-specific band detected at ~102 kDa in mesangial cell lysates. Results shown are representative of those obtained from 3 independent experiments.

Fig. 10. Effects of ANG II and Aldo on mesangial BMP systems. A: total cellular RNAs were extracted from MES13 cells cultured for 24 h in the absence (Control) or presence of ANG II (30 nM) and Aldo (100 nM). Extracted RNAs (2.0 μg) were used as templates for GEArray analysis (SuperArray Bioscience). The signal intensities of the spots on the membranes obtained from 2 separate experiments were analyzed with the GEArray analyzer program (SuperArray Bioscience) after subtraction of the background levels of pUC18 DNA. Cyclophilin A (PPIA) is used as a positive control to compare the membranes. ALK, activin-like kinase. B: after preculture, the cells were treated with or without ANG II (30 nM) and Aldo (100 nM) for 24 h. Total cellular RNA was extracted and ALK-3, ActRII, BMPRII, Smad6, BMP-4, and BMP-7 mRNA levels were examined by real-time RT-PCR. The expression levels of target genes were standardized by RPL19 level in each sample. Results are shown as means ± SE of data from at least 3 separate experiments, each performed with triplicate samples. *P < 0.05, **P < 0.01 vs. each control.
sterone-stimulated mesangial mitosis, p38 activation seems not to be directly involved in mesangial mitogenesis induced by aldosterone.

BMPs have also been shown to signal via the p38 class of MAPK in various cell types (29, 36). In renal tissues, the spatial expression of p38 MAPK in both the ureteric bud and the metanephric blastema has been reported (52). Pharmacological inhibition of p38 in embryonic explants ablates kidney growth and induces marked mesenchymal cell apoptosis (21), suggesting a functional role for p38 signaling in renal development. A recent study by Hu and colleagues (25) demonstrated the interaction between p38 signaling and BMP-dependent stimulation of collecting duct cell morphogenesis in the kidney. In that study, BMP-7 stimulated p38 activity at low doses but it inhibited at high doses, in which ALK-Smad1 signaling negatively regulated the p38 activity (25). Interestingly, BMP-7 had no effect on other MAPK activation including ERK1/2 and SAPK/JNK (25). Given that aldosterone-induced ERK1/2 and SAPK/JNK phosphorylation and mesangial mitosis were, in parallel, suppressed by BMP-4 and BMP-7, the activation of ERK1/2 and SAPK/JNK is most likely involved in aldosterone-induced mesangial cell proliferation compared with the p38 pathway.

In experimental animal models of obstructive or diabetic nephropathy, exogenously administered BMP-7 reduces glomerular and tubulointerstitial fibrosis and preserves renal function (46, 76). BMP-7 treatment can even resolve glomerular and interstitial fibrosis in rats with diabetic nephropathy (76), which results from inhibition of TGF-β effects on fibrosis mediators and matrix protein-degrading enzymes (77). With regard to the underlying mechanism of the beneficial effects of BMP-7, Wang and Hirschberg (77) have shown interesting results that BMP-7 antagonizes TGF-β-induced glomerular fibrogenesis such as connective tissue growth factor expression and accumulation of collagen, fibronectin, and thrombospondin in cultured mouse mesangial cells, suggesting that BMP-7 opposes several profibrogenic activities of TGF-β in mesangial cells. Further investigation revealed that BMP-7-induced inhibition of TGF-β-dependent profibrogenic activities in mesangial cells requires Smad5 and that this action is mediated downstream, at least in large part, through Smad6 (78). Renal TGF-β expression is elevated in renal hypertensive (33) or aldosterone-dependent renal damage shown in the remnant kidney model, and also renal TGF-β production is provoked by aldosterone at posttranscriptional levels (30). Thus BMP effects may play an antagonistic role in aldosterone-induced TGF-β actions leading to amelioration of glomerular sclerosis.

Moreover, BMPs directly inhibit aldosterone-induced MAPK activation, resulting in suppression of mesangial cell mitosis. Nishiyama and his colleagues demonstrated that glomerular cellularity is increased in aldosterone-treated rats (48, 49) and that aldosterone promotes rat mesangial cell proliferation in vitro through activating MAPK (48). MAPK signaling molecules are important mediators of the intracellular signal transduction pathways responsible for cell growth and differentiation in many cell types. Glomerular injury and renal fibrosis induced by aldosterone are likely to be associated with the activation of MAPK pathways including ERK1/2, SAPK/JNK and big-MAPK-1 (49). Considering that aldosterone-induced renal injury and MAPK activation were prevented by concomitant treatment with eplerenone, MAPK molecules are important signaling factors mediating aldosterone-induced renal injury. In rat mesangial cells aldosterone activated ERK1/2 phosphorylation at time points of 5–60 min (48), and both eplerenone and the inhibition of ERK1/2 activation with PD-98059 abolished aldosterone-induced cell proliferation in renal fibroblasts (47). These findings indicate that ERK1/2 could be an important mediator of aldosterone-induced renal injuries.

In addition to its classic genomic actions mediated through regulation of nuclear gene transcription and protein synthesis, aldosterone also elicits rapid, potentially nongenomic, responses in a variety of cells (15). MR antagonists block aldosterone-induced activation of ERK1/2 in VSMCs (28) and Chinese hamster ovary cells transfected with human MR (18), although it is also reported that effects of aldosterone on ERK1/2 activation were not affected by MR antagonists in cortical collecting duct cells (61). In our present study, aldosterone stimulated mesangial cell mitosis in a concentration-dependent manner, which was restored by eplerenone treatment.

Furthermore, eplerenone suppressed aldosterone-induced ERK1/2 and SAPK/JNK phosphorylation in accordance with aldosterone-induced cell mitosis. MR antagonists have been shown to attenuate the rapid aldosterone-induced activation of Kit-RasA (66) and c-Src (9) that leads to ERK1/2 activation in cardiovascular cells. MR antagonists are also able to block several nongenomic actions of aldosterone on vascular Na⁺-K⁺-ATPase (1) and arterial tone (39, 43, 73). In light of these observations, it can be speculated that, in addition to its role as a transcription factor, MR could be involved in a cell signaling system including the ERK1/2 pathway in renal cells. Nevertheless, aldosterone has also been shown to activate MAPK through classic genomic MR-mediated pathways, as reported by other investigators (20). In our present study, aldosterone-induced ERK1/2 phosphorylation was blocked by pretreatment with a transcriptional inhibitor, ACD, and a translational inhibitor, CHX, leading to a conclusion that these aldosterone effects are, at least in part, involved in genomic activation through MR. The molecular mechanism by which BMPs antagonize the MAPK phosphorylation through genomic action by aldosterone needs to be further elucidated.

The normal physiological actions of aldosterone on mesangial cells have yet to be elucidated. Transcripts for the MR have been detected in renal glomeruli, albeit at lower levels than in the distal tubular epithelium (72). Assuming that glomerular MR were confined to mesangial cells (~15% of glomerular volume), the density of MR could be essentially similar to mesangial MR. Given the finding that aldosterone stimulates type IV collagen synthesis by mesangial cells in vitro (75), mesangial MR may mediate fibrogenesis and sclerosis in the glomeruli. In this regard, Nishiyama et al. (48) reported that mesangial cells readily respond to aldosterone by reducing the elastic modulus as shown by a nanoidentation technique. Reduction of the elastic modulus of endothelial cells was shown to attenuate the rapid aldosterone-induced activation of Ki-RasA (66) and c-Src (9) that leads to ERK1/2 activation in cardiovascular cells. MR antagonists are also able to block several nongenomic actions of aldosterone on vascular Na⁺-K⁺-ATPase (1) and arterial tone (39, 43, 73). In light of these observations, it can be speculated that, in addition to its role as a transcription factor, MR could be involved in a cell signaling system including the ERK1/2 pathway in renal cells. Nevertheless, aldosterone has also been shown to activate MAPK through classic genomic MR-mediated pathways, as reported by other investigators (20). In our present study, aldosterone-induced ERK1/2 phosphorylation was blocked by pretreatment with a transcriptional inhibitor, ACD, and a translational inhibitor, CHX, leading to a conclusion that these aldosterone effects are, at least in part, involved in genomic activation through MR. The molecular mechanism by which BMPs antagonize the MAPK phosphorylation through genomic action by aldosterone needs to be further elucidated.

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physiological role in maintaining mesangial morphology in the glomeruli.

In the present study, the ANG II effect was a key factor in the growth response of this cell line. In the kidney, it is reported that ANG II stimulates proliferation of cultured human and murine mesangial cells (3, 80). In contrast, cells undergoing ANG II-mediated hypertrophy have also been demonstrated in renal tubular cells, in which ANG II induction of p27kip1 expression is related to the mechanism of glomerular cell hypertrophy in porcine proximal tubular LLC-PK1 cells (81), mouse proximal tubular cells, and VSMCs (5). In the current mesangial cell line, cellular hypertrophy in response to ANG II was not detectable microscopically in serum-free cultured conditions. This mesangial cell line is originated from the kidney of transgenic mice of large T antigen in the early region of SV40, in which the renal phenotype shows glomerulosclerosis and proliferative tubular lesions with multiple tubular cysts (41). These findings imply that cell cycle regulation is accelerated or distinct from normal mesangial cells. However, the absence of T antigen expression by glomerular cells and the presence of differential growth pattern among endothelial, epithelial, and mesangial cells suggest that extraglomerular factors are possibly involved in the pathogenesis of glomerular lesions and that the normal regulatory machinery for glomerular cell proliferation is still preserved. Actually, mesangial cell proliferation is arrested in serum-free conditions although it occurs more rapidly than normal cells in the presence of serum (40). Nevertheless, since the mitotic response to aldosterone could be specific to this mesangial cell line, we need to expand our findings regarding the signal interaction between aldosterone and BMPs to primary cell culture study and/or in vivo situation in future studies.

Collectively, BMP-4 and BMP-7 antagonize aldosterone-induced mesangial cell proliferation through inhibiting MAPK activation as well as MR expression. These results suggest the existence of a novel signal cross talk between the BMP system and aldosterone signaling through MAPK in renal cells. On the other hand, aldosterone not only decreases endogenous BMP-4 and BMP-7 but also impairs Smad1, -5, and -8 signal activation by inhibiting ALK-3 (BMPRIA) expression and upregulating Smad6 expression in mesangial cells. Thus a functional link between circulating the ANG II-aldosterone system and the mesangial BMP system was uncovered here.

ACKNOWLEDGMENTS

We thank Dr. R. Kelly Moore for helpful discussion and critical reading of the manuscript. We also thank Drs. Tetsuro Watabe and Kohei Miyazono for providing Id-1-Luc plasmid.

GRANTS

This work was supported in part by Grants-in-Aid for Scientific Research, Yamaguchi Endocrine Research Association, Life Science Foundation of Japan, Takeda Science Foundation, The Tokyo Biochemical Research Foundation, Ryohei Teien Memory Foundation, Sakakibara Memorial Research Grant, The Kawasaki Foundation for Medical Science & Medical Welfare, and The Ichiro Kanehara Foundation.

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