Aldosterone Stimulates Vascular Smooth Muscle Cell Proliferation Via Big Mitogen-Activated Protein Kinase 1 Activation

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Abstract—The nongenomic effects of aldosterone have been implicated in the pathogenesis of various cardiovascular diseases. Aldosterone-induced nongenomic effects are attributable in part to the activation of extracellular signal-regulated kinase 1/2 (ERK1/2), a classical mitogen-activated protein (MAP) kinase. Big MAP kinase 1 (BMK1), a newly identified MAP kinase, has been shown to be involved in cell proliferation, differentiation, and survival. We examined whether aldosterone stimulates BMK1-mediated proliferation of cultured rat aortic smooth muscle cells (RASMCs). Mineralocorticoid receptor (MR) expression and localization were evaluated by Western blotting analysis and fluorolabeling methods. ERK1/2 and BMK1 activities were measured by Western blotting analysis with the respective phosphospecific antibodies. Cell proliferation was determined by Alamar Blue colorimetric assay. Aldosterone (0.1 to 100 nmol/L) dose-dependently activated BMK1 in RASMCs, with a peak at 30 minutes. To clarify whether aldosterone-induced BMK1 activation is an MR-mediated phenomenon, we examined the effect of eplerenone, a selective MR antagonist, on aldosterone-induced BMK1 activation. Eplerenone (0.1 to 10 μmol/L) dose-dependently inhibited aldosterone-induced BMK1 activation in RASMCs. Aldosterone also stimulated RASMC proliferation, which was inhibited by eplerenone. Aldosterone-mediated phenomena were concluded to be attributable to a nongenomic effect because cycloheximide failed to inhibit aldosterone-induced BMK1 activation. Transfection of dominant-negative MAP kinase/ERK kinase 5 (MEK5), which is an upstream regulator of BMK1, partially inhibited aldosterone-induced RASMC proliferation, which was almost completely inhibited by MEK inhibitor PD98059. In addition to the classical steroid activity, rapid nongenomic effects induced by aldosterone may represent an alternative etiology for vascular diseases such as hypertension. (Hypertension. 2005;46[part 2]:1046-1052.)

Key Words: mineralocorticoids ■ aldosterone ■ protein kinases

Accumulating evidence suggests that aldosterone plays an important role in the pathogenesis of various cardiovascular diseases, including hypertension.1,2 Aldosterone is currently thought to be a more common cause of hypertension than believed previously and has been shown to induce vasoconstriction, endothelial dysfunction, and myocardial fibrosis, in part through the activation of angiotensin II-dependent pathways.3,4 In terms of classical genomic activity, aldosterone has been shown to exert effects via intracellular mineralocorticoid receptors (MRs), a superfamily of ligand-regulated transcription factors, to regulate blood pressure and electrolyte balance through interaction with the regulatory region of target gene promoters.5,6

Nevertheless, aldosterone signaling is not solely a genomic event, and rapid nongenomic effects in a variety of tissues, including vascular smooth muscle cells (VSMCs), have been reported.7,8 The rapid nongenomic effects of aldosterone are characterized by a rapid onset of action within minutes, an insensitivity to inhibitors of protein synthesis such as cycloheximide, and an insensitivity to classical MR antagonist.9 Aldosterone induces rapid responses in VSMCs, such as increases in intracellular Ca2+ and cAMP levels, as well as activation of protein kinase C and the Na+ /H+ exchanger.5,9 Aldosterone also causes phosphorylation of various intracellular signaling molecules including c-Src,10 epidermal growth factor receptor,11 and mitogen-activated protein (MAP) kinases, such as extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun NH2-terminal kinase (JNK), and p38.10,11,12 We also reported that aldosterone stimulates ERK1/2 activation in cultured rat mesangial cells.13 The activation of these MAP kinase pathways has been shown to be involved in the promotion of VSMC proliferation and the hypertrophy that relates to the vascular remodeling and altered tone in hypertension.14

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In addition to the classical MAP kinases (ie, ERK1/2, JNK, and p38), big MAP kinase 1/ERK 5 (BMK1/ERK5) was identified recently as a new MAP kinase family member with a large COOH-terminal and a unique loop-12 sequence that shares the TEY activation motif with ERK1/2 but is activated by MAP kinase/ERK kinase 5 (MEK5).\textsuperscript{15} Although an important role for BMK1 in concentric cardiac hypertrophy and endothelial survival in the maintenance of vascular integrity has been reported,\textsuperscript{16,17} a role for BMK1 in hypertension has not been reported yet, and aldosterone-induced BMK1 activation in VSMCs still remains to be demonstrated. BMK1 has been shown to be activated by several intracellular molecules including reactive oxygen species, c-Src, and protein kinase C,\textsuperscript{18,19} all of which are also activated by aldosterone stimulation in VSMCs.\textsuperscript{9,10} Recently, we also demonstrated that BMK1 is activated in the glomeruli of diabetic rats, and that a high concentration of glucose stimulates BMK1 activation and proliferation of rat mesangial cells.\textsuperscript{19} Therefore, we hypothesized that BMK1 may be activated by aldosterone in VSMCs and play some role in hypertension.

In the present study, we first examined the effect of aldosterone on changes in BMK1 activity, as well as ERK1/2 activity, in cultured rat aortic smooth muscle cells (RASMCs). The effect of aldosterone on RASMC proliferation was also examined using colorimetric measurement with Alamar Blue assay. Thereafter, we examined the effect of eplerenone, a selective MR antagonist, on aldosterone-induced BMK1 activation and RASMC proliferation. The effects of cycloheximide and tiron on aldosterone-induced BMK1 activation were also examined. Finally, we examined the effect of transfection of dominant-negative MEK5 (DN-MEK5), which is an upstream regulator of BMK1, on aldosterone-induced RASMC proliferation to distinguish the involvement of BMK1 from ERK1/2 in aldosterone-mediated intracellular signaling pathways.

Materials and Methods

Cell Culture

Cultured RASMCs were prepared according to published methods (please see the online data supplement, available at http://www.hypertensionaha.org).\textsuperscript{20} Cells were used for experiments after 48-hour serum starvation. Control solutions always contained the appropriate amount of vehicle (ethanol for aldosterone [Across Organics], dimethylsulfoxide for eplerenone [Pfizer Inc.] and PD98059 [Merck KGaA], and distilled water for cycloheximide [Sigma Chemical Co.], respectively) at concentrations of 100 μmol/L. After stimulation, protein or mRNA was extracted as described previously.\textsuperscript{21} Protein concentrations were determined using Bradford protein assay kits (Bio-Rad Laboratories).

Western Blotting Analysis

MR protein expression was determined by Western blotting analysis with MR-specific antibody (Santa Cruz Biotechnology) as described previously.\textsuperscript{22} To evaluate activated ERK1/2 and BMK1/ERK5, immunoblotting was performed with antibodies against phospho-ERK1/2 and phospho-BMK1/ERK5 (Cell Signaling Technology) as described.\textsuperscript{19–21} In addition, the total ERK1/2 and BMK1/ERK5 protein expressions were measured using a pan-ERK1/2 antibody and pan-BMK1 antibody (Cell Signaling Technology).\textsuperscript{19–21}

Immunofluorescence

The multiple fluorolabeling methods used generally followed those in our previous study.\textsuperscript{26} RASMCs were fixed and stained with polyclonal anti-MR and monoclonal anti–α1-integrin antibodies and were subjected to secondary fluorolabeling. α1-Integrin was used as a marker for the cell membrane.\textsuperscript{22} RASMC nuclei were also stained with propidium iodide (PI). RASMCs double-stained with the above-mentioned combinations of antibodies were then examined using a confocal laser scanning light microscope (TCS-NT; Leica).

Real-Time RT-PCR

The MR mRNA expression levels were analyzed by real-time PCR as described previously.\textsuperscript{11} The primer sequences for MR amplification were 5′-gctcatgctgtagtacg-3′ and 5′-aagttcttcctggccggtat-3′. All data were normalized by the expression of GAPDH. The primer for GAPDH was synthesized on the basis of published sequences.\textsuperscript{21}

Alamar Blue Assay for Cell Proliferation Evaluation

RASMC proliferation was measured with an Alamar Blue assay according to manufacturer protocol (Trek Diagnostics Systems Inc.). Briefly, Alamar Blue was diluted 1 to 10 in the cell culture media, and the color change was monitored. Colorimetric evaluation of cell proliferation was performed using a spectrophotometer with 540 nm as excitation wavelength and 590 nm as emission wavelength. Cells were stimulated for 24 hours with 100 nmol/L of aldosterone. Eplerenone and PD98059 were added to the incubation medium 30 minutes before aldosterone stimulation, respectively.

Transfection of DN-MEK5

The DN-MEK5 construct subcloned into pcDNA3.1 was provided by Dr Eisuke Nishida of Kyoto University.\textsuperscript{23} Transfections of the cells were performed using the Nucleofector kit (Amaxa Biosystems) as described previously.\textsuperscript{20} Transfection efficiency was determined with pcDNA3.1–green fluorescent protein transfection to be ≈45% in RASMCs. Two days after transfection, RASMCs were subjected to cell proliferation assay.

Statistical Analysis

Values are presented as the mean±SE. One-way ANOVA was used to determine significance among groups, after which a modified t test with the Bonferroni correction was used for comparison between individual groups. P<0.05 was considered statistically significant.

Results

Expression of MR in RASMCs

As shown in Figure 1, Western blotting analysis with the MR-reactive antibody yielded a prominent band of ≈110 kDa in total lysates of RASMCs. Further, the observed band was displaced when immunoblotting was performed in the presence of the primary antibody and the peptide fragment of the MR used to generate the primary antibody (Santa Cruz Biotechnology; Figure 1). Similarly, real-time PCR analysis revealed significant gene expression of MR in RASMCs (n=4; data not shown). These results make evident the presence of MR in VSMCs at the DNA and protein level.

To confirm the localization of MR in RASMCs, we performed studies using fluorolabeling methods. As shown in Figure 2, MR staining (green) was observed in the cytoplasm as well as in the nucleus of RASMCs. Superimposing the images did not reveal any areas of colocalization of MR and the known membrane marker α1-integrin (blue), suggesting that expression of MR was not expressed in the cell membrane of RASMCs.
Aldosterone-Induced BMK1 and ERK1/2 Activation in RASMCs
We examined whether BMK1 was activated by aldosterone in RASMCs. Growth-arrested RASMCs were treated at various times and concentrations of aldosterone. As shown in Figure 3A, aldosterone (100 nmol/L) treatment of cells resulted in a rapid rise in the activation of BMK1 within 10 minutes, which peaked at 30 minutes and then declined. Figure 3B shows the concentration-dependent effects of aldosterone treatment (30 minutes) on the BMK1 activity (n=5 for each). Aldosterone stimulated concentration-dependent BMK1 activation, and maximal activation occurred at 100 nmol/L of aldosterone. On the other hand, no significant differences in the amounts of total BMK1 were demonstrated in samples by Western blotting analysis with anti–pan-BMK1 antibody (Figure 3A and 3B).

In addition to BMK1 activation, we also examined whether aldosterone activates ERK1/2 in RASMCs. As shown in Figure 4A, aldosterone significantly activated ERK1/2, with a peak at 20 minutes (n=5). At this time point, aldosterone caused ERK1/2 activation in a concentration-dependent manner (Figure 4B). No significant differences in the amounts of total ERK1/2 were demonstrated in samples by Western blotting analysis with anti–pan-ERK1/2 antibody (Figure 4A and 4B).

Effects of Cycloheximide, PD98059, Tiron, and Eplerenone on Aldosterone-Induced BMK1 Activation in RASMCs
To investigate the role of protein synthesis in aldosterone-induced stimulation of BMK1, we preincubated RASMCs with cycloheximide (10 μg/mL) for 15 minutes. Cycloheximide had no effect on aldosterone-induced BMK1 activation at 30 minutes (n=5 for each; Figure 5A). On the other hand, preincubation with PD98059 (100 μmol/L for 30 minutes), a MEK1/2 inhibitor and also known as an inhibitor of MEK5, which is an upstream activator of BMK1/ERK5, prevented aldosterone-induced BMK1 activation (n=5). An oxygen radical scavenger, tiron (10 μmol/L for 30 minutes) also inhibited aldosterone-induced BMK1 activation (n=5). Similar results were obtained in the case of ERK1/2 activation, in which PD98059 and tiron, but not cycloheximide, inhibited aldosterone-induced ERK1/2 activation (n=5 for each; Figure 5A). The doses and preincubation times of cycloheximide, PD98059, and tiron were determined on the basis of results from previous in vitro studies.

Figure 1. MR protein expression in RASMCs measured by Western blotting analysis. Significant MR protein expression is detected at ~110 kDa in total lysates of RASMCs. The band is not present when incubating the blot that was incubated with a mixture of the primary antibody and the antigenic-specific peptides. IB indicates immunoblotting.

Figure 2. Confocal laser microscopic observation of the MR and α1-integrin distribution in RASMCs. Green and blue fluorescence represent MR and α1-integrin staining, respectively. RASMC nuclei were also stained with PI. Superimposition of the images does not reveal any areas of colocalization of MR and α1-integrin (a known plasma membrane marker), whereas a certain amount of MR localization in the nucleus was observed.
To investigate the role of MR in aldosterone-induced BMK1 activation, the effects of the selective MR antagonist eplerenone on BMK1 activity were examined. RASMCs were pretreated with eplerenone (0.1 to 10 \( \mu \text{mol/L} \)) for 30 minutes before stimulation with aldosterone (100 nmol/L). As shown in Figure 5B, eplerenone attenuated aldosterone (30 minutes)-stimulated BMK1 activity in a concentration-dependent manner (n=5 for each).

Aldosterone-Induced RASMC Proliferation and Its Inhibition by Eplerenone, PD98059, and Transfection of DN-MEK5

The effects of aldosterone on RASMC proliferation were determined by Alamar Blue assay (n=5 for each). Aldosterone treatment for 30 hours significantly increased RASMC proliferation (147±12% of controls; Figure 6A). Aldosterone-induced increases in cell proliferation were significantly inhibited by preincubation with eplerenone (0.1 to 10 \( \mu \text{mol/L} \)) for 30 minutes before stimulation with aldosterone (100 nmol/L). As shown in Figure 5B, eplerenone attenuated aldosterone (30 minutes)-stimulated BMK1 activity in a concentration-dependent manner (n=5 for each).

However, because it has been reported that VSMC proliferation is augmented by the activation of ERK1/2,12,14 which is also inhibited by PD98059, an involvement of ERK1/2 in aldosterone-induced cell proliferation cannot be ruled out. We also observed that PD98059 inhibited aldosterone-induced ERK1/2 activation to an extent similar to BMK1 activation (Figure 5A). Therefore, to separate the involvement of BMK1 from that of ERK1/2 in aldosterone-induced cell proliferation, we used cells transfected with a plasmid of DN-MEK5. As shown in Figure 6A, transfection with DN-MEK5 significantly inhibited aldosterone-induced RASMC proliferation compared with that of control cells; however, the inhibitory effect of DN-MEK5 transfection on aldosterone-induced cell proliferation was less potent than the inhibition by PD98059 treatment. The specificity and efficacy of the BMK1 inhibition by DN-MEK5 transfection was evaluated by Western blotting analysis with MEK5, phospho-ERK1/2, and phospho-BMK1/ERK5 antibodies, respectively (Figure 6B).

Discussion

It is now widely recognized that aldosterone has rapid nongenomic effects in a variety of tissues, including VSMCs,8,9 and that MR is expressed in a variety of nonepithelial cells, such as VSMCs,24 cardiomyocytes,25 and mononuclear leukocytes.26 Therefore, we first examined the expression of MR in RASMCs using several different procedures, such as Western blotting, real-time PCR, and immunofluorescent staining. A significant expression of an MR protein with a band of \( \approx \) 110 kDa, which corresponds to the approximate molecular weight of rat MR in tissues, was demonstrated in RASMCs by Western blotting analysis with an MR-specific antibody that corresponds to the
no obvious colocalization of MR with other NRs present predominantly in the cytoplasm of RASMCs because confocal laser microscopic observations revealed that MR is examined the subcellular localization of MR in RASMCs.

With real-time PCR analysis, we found that aldosterone-stimulated MR expression in RASMCs.27 We also observed that the band did not appear after incubating the blot with a mixture of the primary antibody and the antigenic-specific peptides (Figure 1), confirming the MR-specific immunoreactivity in the Western blot. These results are consistent with the very recent findings of Jaffe and Mendelsohn, who demonstrated the existence of functional MR in human VSMCs.28 In addition to the confirmation of MR gene expression in RASMCs with real-time PCR analysis, we examined the subcellular localization of MR in RASMCs. Confocal laser microscopic observations revealed that MR is present predominantly in the cytoplasm of RASMCs because no obvious colocalization of MR with α1-integrin, a plasma membrane marker, was observed. PI staining of the nucleus revealed the localization of a certain amount of MR in the nucleus. Together, these results, obtained by different experimental procedures, clearly support the presence of MR in RASMCs.

MAP kinases are important mediators of the intracellular signal transduction pathways responsible for cell growth and differentiation.13,14 In VSMCs, rapid aldosterone-induced activation of ERK1/2 has been reported.29 Previously, we also reported that aldosterone-induced ERK1/2 activation mediates the proliferation of cultured rat mesangial cells.13 These findings suggest that ERK1/2 is an important mediator of aldosterone-induced cell proliferation. However, the physiological role of the new MAP kinase family member BMK1 in VSMCs has not been well documented, and the effect of aldosterone on BMK1 activity has not been demonstrated previously. We observed for the first time that BMK1 is activated by aldosterone in RASMCs, and the activation occurred within 10 minutes, suggesting a nongenomic effect. We reported previously a rapid and significant activation of BMK1 attributable to high-concentration glucose stimulation in rat mesangial cells.19 Because it was reported that PD98059, a MEK1/2 inhibitor, also inhibits MEK5, which is an upstream regulator of BMK1,23,24 we examined the effect of PD98059 on aldosterone-induced BMK1 activation. We found that PD98059 significantly inhibited aldosterone-induced BMK1 activation as well as ERK1/2 activation in RASMCs (Figure 5A). We also observed that cycloheximide had no effect on aldosterone-induced BMK1 activation. These results indicate that the activation of BMK1 induced by aldosterone is independent of transcription and translation, and are likely, therefore, to be mediated through nongenomic mechanisms. Because an antioxidant tiron inhibited aldosterone-induced BMK1 activation, oxidative stress may also be involved in its mechanism. The present study demonstrates that preincubation with eplerenone significantly attenuated rapid aldosterone-induced BMK1 activation in RASMCs. In this regard, recent studies have indicated that some of the rapid actions of estrogen and other steroid hormones, including aldosterone, are mediated by intracellular steroid receptors.12,30 For example, Mazak et al12 showed that aldosterone potentiated angiotensin II–induced rapid ERK1/2 activation in VSMCs, and that this effect was abolished by treatment with spironolactone. Similarly, aldosterone-induced rapid activation of Ki-RasA, an upstream activator of the BMK1 cascade,31 was markedly attenuated by spironolactone.32 In addition, MR antagonists are able to block several nongenomic actions of aldosterone on vascular Na+,K+-ATPase,9 arterial tone,33 as well as Src and p38 kinase activation.10 On the basis of these observations, along with the results of the present study, we hypothesize that in addition to its role as a transcription factor, MR could be involved in cell-signaling systems, including the BMK1 pathway in RASMCs.

It was reported previously that aldosterone induced an increase in [3H]proline, an index of collagen synthesis, in VSMCs.10 It has also been reported that aldosterone stimulates proliferation of other types of cells, such as cardiac fibroblasts12 and mesangial cells.13 In agreement with these previous observations, the present study reveals that treat-
suggest that ERK1/2 and BMK1 are involved in the aldosterone-induced RASMC proliferation in parallel ways. Because the cellular proliferative effect of aldosterone was prevented by eplerenone, aldosterone may induce these cellular phenotypes via the activation of MR in RASMCs.

**Perspectives**

The in vitro and animal data support a direct role for mineralocorticoids in the regulation of vascular function and VSMC proliferation. Spironolactone is known to be able to directly dilate isolated rat aortic rings, and stimulation of VSMC proliferation by angiotensin II is inhibited by spironolactone as well as other aldosterone antagonists. In rats with aldosterone-induced hypertension, another aldosterone antagonist, eplerenone, decreases carotid artery cross-sectional thickness. These findings are consistent with the present result that MR is involved in aldosterone-induced RASMC proliferation, indicating that VSMCs are one of the major targets for aldosterone. However, the intracellular signaling mechanisms that mediate VSMC proliferation are still largely unknown. In the present study, we observed for the first time that BMK1, a new MAP kinase, may be involved in the pathogenesis of aldosterone/MR-induced vascular injury. Elucidation of the MR-mediated intracellular signaling pathways, including BMK1, will provide information vital to the development of new drugs for aldosterone-dependent cardiovascular injury. Further studies to elucidate the precise molecular mechanisms by which aldosterone mediates vascular injury via activation of MR are under way.

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**References**


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