Involvement of Aldosterone and Mineralocorticoid Receptors in Rat Mesangial Cell Proliferation and Deformability

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Abstract—We demonstrated recently that chronic administration of aldosterone to rats induces glomerular mesangial injury and activates mitogen-activated protein kinases including extracellular signal-regulated kinases 1/2 (ERK1/2). We also observed that the aldosterone-induced mesangial injury and ERK1/2 activation were prevented by treatment with a selective mineralocorticoid receptor (MR) antagonist, eplerenone, suggesting that the glomerular mesangium is a potential target for injuries induced by aldosterone via activation of MR. In the present study, we investigated whether MR is expressed in cultured rat mesangial cells (RMCs) and involved in aldosterone-induced RMC injury. MR expression and localization were evaluated by Western blotting analysis and fluorolabeling methods. Cell proliferation and micromechanical properties were determined by [3H]-thymidine uptake measurements and a nanoindentation technique using an atomic force microscope cantilever, respectively. ERK1/2 activity was measured by Western blotting analysis with an anti-phospho–ERK1/2 antibody. Protein expression and immunostaining revealed that MR was abundant in the cytoplasm of RMCs. Aldosterone (1 to 100 nmol/L) dose-dependently activated ERK1/2 in RMCs with a peak at 10 minutes. Pretreatment with eplerenone (10 \text{nmol/L}) significantly attenuated aldosterone-induced ERK1/2 phosphorylation. Aldosterone (100 nmol/L) treatment for 30 hours increased [3H]-thymidine incorporation and decreased the elastic modulus, indicating cellular proliferative and deforming effects of aldosterone, respectively. These aldosterone-induced changes in cellular characteristics were prevented by pretreatment with eplerenone or an ERK (MEK) inhibitor, PD988059 (100 \text{nmol/L}). The results indicate that aldosterone directly induces RMC proliferation and deformability through MR and ERK1/2 activation, which may contribute to the pathogenesis of glomerular mesangial injury. (Hypertension. 2005;45[part 2]:710-716.)

Key Words: mineralocorticoids ■ aldosterone
induced by aldosterone via activation of MR. However, the expression of MR and the cellular actions of aldosterone in the glomerular mesangium have not been investigated. Therefore, the present study aimed to determine whether MR is expressed in cultured rat mesangial cells (RMCs) and involved in aldosterone-induced RMC injury. Because aldosterone possesses stimulatory effects on the proliferation of cardiac fibroblasts, vascular smooth muscle cells, and Madin–Darby canine kidney cells, the effects of aldosterone and MR blockade on glomerular cell proliferation were examined. We also investigated the micromechanical properties of living glomerular cells by a nanoindentation technique using an atomic force microscope cantilever. In aldosterone-treated rats, glomerular mesangial injury was associated with the activation of mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinases 1/2 (ERK1/2). Therefore, the role of ERK1/2 in the mesangial cellular actions of aldosterone was also investigated.

Materials and Methods

Cell Culture

All experimental procedures were performed according to the guidelines for the care and use of animals established by Kagawa Medical University. RMCs were isolated from male Sprague-Dawley rats and maintained according to published methods.

Western Blotting Analysis

MR protein expression was determined by Western blotting analysis with MR-specific antibody (Santa Cruz Biotechnology), as described previously. To check for equal loading, membranes were reprobed with an antibody against β-actin (Sigma). To evaluate activated ERK1/2, immunoblotting was performed with antibodies against phospho-ERK1/2 (Cell Signaling Technology) as described. In some RMCs, nuclear and membrane fractions were isolated as described. Protein concentrations were determined using Bradford or Lowry protein assay kits (Bio-Rad).

Confocal Microscopy

The multiple fluorolabeling methods used generally followed those in our earlier study. Briefly, RMCs were incubated with a mixture of anti-MR (1:200) and anti–α1-integrin (1:200) antibodies overnight at 4°C. Mouse monoclonal anti–α1-integrin was a gift from Dr Shoji Kagami (The University of Tokushima School of Medicine, Japan). α1-Integrin was used as a marker for the cell membrane. Thereafter, RMCs were incubated in a mixture of species-specific secondary antibodies, fluorescein isothiocyanate–conjugated donkey anti-rabbit IgG, and Cy3-conjugated donkey anti-mouse IgG (1:200; Jackson Immunoresearch), respectively, for 1 hour. RMCs double-stained with the above-mentioned combinations of fluoroprobe 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. Briefly, the amplification protocol consisted of 1 incubation at 95°C for 10 minutes to activate the polymerase, and then 40 cycles of PCR (95°C for 10 s, 60°C for 5 s, and 72°C for 8 s). The primer sequences for MR amplification were 5'-tgcagctcggatctgac-3' and 5'-aagtccctgccggtat-3'. All data were normalized by the expression of GAPDH. The primer for GAPDH was synthesized based on published sequences.

[3H]-Thymidine Incorporation and Mechanical Properties

Cell proliferation was determined by [3H]-thymidine incorporation as described previously. To determine the mechanical properties of RMCs, the relationship between the cantilever deflection and its indentation depth was obtained by pressing the atomic force microscope cantilever (NVB 100; Olympus) into the cell surface as described previously. The elastic modulus was estimated using the Hertz model, which describes the indentation of a homogeneous/semi-infinite elastic material.

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 RMCs

As shown in Figure 1A, Western blotting analysis with the MR-reactive antibody yielded a prominent band at ~110 kDa in total lysates of RMCs. 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 1A). Equal amounts of protein were transferred, and no differences in the amounts of β-actin were observed in these samples (data not shown). Similarly, real-time PCR analysis revealed significant gene expression of MR in RMCs (n=4; data not shown).

In RMCs, a certain amount of MR protein expression was observed in the nuclear fraction (Figure 1B) but was barely detectable in the membrane fraction (Figure 1C). On the other hand, abundant MR protein expression was observed in the total lysates, indicating that MR is predominantly present in the cytoplasm of RMCs. These results were confirmed by studies using fluorolabeling methods. As shown in Figure 2, MR staining (green) was predominantly observed in the cytoplasm of RMCs and was lower in the nucleus. Superimposing the images did not reveal any areas of colocalization of MR and the known membrane marker α1-integrin (red), suggesting no expression of MR in the cell membrane of RMCs.

ERK1/2 Phosphorylation

Figure 3A shows the concentration–dependence effects of aldosterone treatment (10 minutes) on the ERK1/2 activity (n=7 to 10 for each). Aldosterone-stimulated ERK1/2 activation was maximal at 100 nmol/L. Figure 3B shows the time course of aldosterone-stimulated ERK1/2 activity (n=6 to 11 for each). Aldosterone (100 nmol/L)-induced activation of ERK1/2 peaked at 10 minutes (3.9±0.8-fold; Figure 3B), and continued for 60 minutes (n=7 to 9 for each). On the other hand, no significant differences in the amounts of total (phosphorylated and unphosphorylated) ERK1/2 were ob-
ERK1/2 activity in a concentration-dependent manner (n = 4 to 6 for each; Figure 4B). On the other hand, preincubation with PD98059 (100 μmol/L for 15 minutes), a specific inhibitor of MAPK/MEK that is the upstream activator of ERK/MAPK, prevented aldosterone-induced ERK1/2 phosphorylation (n = 4). The doses and preincubation times of actinomycin D, cycloheximide and PD98059 were determined on the basis of results from previous in vitro studies.19,23–25

Cell Proliferation and Micromechanical Properties
Effects of aldosterone on cell proliferation were determined by [3H]-thymidine incorporation (n = 4 to 8 for each). Treatment with aldosterone (100 nmol/L) for 15 hours did not alter [3H]-thymidine incorporation in RMCs (data not shown). However, aldosterone treatment for 30 hours significantly increased [3H]-thymidine incorporation (133 ± 8% of controls; Figure 5A). Aldosterone-induced increases in [3H]-thymidine incorporation were significantly inhibited by preincubation with eplerenone (10 μmol/L; 110 ± 11% of controls) or PD98059 (100 μmol/L; 105 ± 15% of controls). However, thymidine incorporation tended to be increased by 45-hour exposure of aldosterone but not statistically significantly (data not shown).

Aldosterone (100 nmol/L) treatment for 30 hours markedly decreased the elastic modulus in living RMCs (47 ± 3% of controls; n = 10; Figure 5B), and these decreases were abolished by preincubation with eplerenone (10 μmol/L; 91 ± 5% of controls; n = 10) or PD98059 (100 μmol/L; 89 ± 5% of controls; n = 10).

Discussion
MR is expressed in a variety of nonepithelial cells, such as vascular smooth muscle cells,26 cardiomyocytes,26 and mononuclear leukocytes.27 In the present study, we investigated the expression of MR in RMCs using several methods. Western blotting analysis with MR-specific antibody detected significant MR protein expression in RMCs with a band of ~110 kDa, which corresponds to the approximate molecular weight of rat MR in tissues.21,22 Similar results were obtained using another MR-specific antibody (data not shown). Further studies showed that the band was not present when incubating the blot with a mixture of the primary antibody and the antigenic-specific peptides (Figure 1A), indicating the MR-specific immunoreactivity on Western blot.21,22 In addition, real-time PCR analysis revealed significant gene expression of MR in RMCs. We also investigated the subcellular localization of MR. A certain amount of MR protein expression was observed in the nuclear fraction, whereas MR protein expression in the membrane fraction was barely detectable. On the other hand, abundant MR protein expression was observed in the total lysates, indicating that MR is predominantly present in the cytoplasm of RMCs. Similar results were obtained in the fluorescent labeling experiments using confocal microscopy (Figure 2). Further, preliminary fluorescent labeling experiments showed that MR translocation from the cytoplasm to the nucleus was induced in RMCs by treatment with aldosterone (Nishiyama and Abe, unpublished data).
2004). Collectively, these data obtained by different experimental approaches support the presence of MR in RMCs.

In aldosterone-treated rats, glomerular cellularity is markedly elevated.\textsuperscript{12} In agreement with previous studies in other cells,\textsuperscript{13–16} the present study showed that treatment with aldosterone for 30 hours increased [\textsuperscript{3}H]-thymidine uptake in RMCs. Determination of the micromechanical properties of living RMCs revealed that RMCs markedly responded to aldosterone by reducing the elastic modulus, indicating that aldosterone has cellular-deforming effects. The significance of this effect of aldosterone is not clear from the present experiments, but recent studies have indicated that reductions in the elastic modulus of endothelial cells facilitate the migration of monocytes.\textsuperscript{17} Furthermore, Blasi et al\textsuperscript{2} showed that glomerular injury and fibrosis were associated with macrophage infiltration in aldosterone-treated rats. Thus, it is interesting to speculate that aldosterone-induced RMC deformation is involved in the migration of cells infiltrating the glomerulus. Because the cellular proliferative and deforming effects of aldosterone were prevented by eplerenone, aldosterone may induce these cellular changes via activation of MR in RMCs.

In addition to its classical genomic actions mediated through regulation of nuclear transcription and protein synthesis, aldosterone also elicits rapid, potentially nongenomic, cellular responses in a variety of cells.\textsuperscript{13,15,16,24,25,28} Therefore, further studies were conducted to examine the effects of actinomycin D (an inhibitor of nuclear transcription\textsuperscript{24,25}) and cycloheximide (an inhibitor of protein synthesis\textsuperscript{24,25}) on the aldosterone-induced RMC changes. However, exposure to both compounds for \textgtr 12 hours resulted in severe damage to RMCs even at very low concentrations (<0.1 \(\mu\)g/L, respectively), and hence, we were unable to perform these experiments. Thus, whether the aldosterone-induced cellular actions in RMCs are mediated by genomic or nongenomic effects of MR could not be addressed in the present study.

**Figure 2.** Distribution of MR and \(\alpha_1\)-integrin in RMCs. Green and red fluorescence represents MR and \(\alpha_1\)-integrin staining, respectively. Superimposition of the images does not reveal any areas of colocalization of MR and \(\alpha_1\)-integrin (a known membrane marker).

**Figure 3.** A, Aldosterone stimulates ERK1/2 activity in RMCs in a concentration-dependent manner. RMCs were stimulated with the indicated concentrations of aldosterone (1 to 1000 nmol/L) for 10 minutes. B, Time course of aldosterone-stimulated ERK1/2 activity in RMCs. RMCs were stimulated with 100 nmol/L aldosterone for the indicated times (5 to 360 minutes; \(n=7\) to 9 for each). The results were normalized by arbitrarily setting the densitometry of the control cells (time=0) to 1.0. *\(P<0.05\) vs control.
MAPKs are important mediators of the intracellular signal transduction pathways responsible for cell growth and differentiation. We observed previously that glomerular mesangial injury was associated with the activation of MAPKs, including ERK1/2, in aldosterone-treated rats. These data suggest that ERK1/2 is an important signaling molecule mediating aldosterone-induced glomerular injury. Consistent with the data obtained in other cells, the present study showed that aldosterone activated ERK1/2 in RMCs. We further observed that inhibition of the ERK1/2 cascade with PD98059 abolished aldosterone-induced ERK1/2 phosphorylation at 10 minutes (n=4 to 6 for each). On the other hand, PD98059 prevents aldosterone-induced ERK1/2 phosphorylation (n=4). V indicates vehicle (0.01% ethanol).

Figure 4. A, Effects of eplerenone on aldosterone-induced ERK1/2 activation in RMCs. Preincubation with eplerenone attenuates aldosterone (10 minutes)-stimulated ERK1/2 activity in a concentration-dependent manner (n=6 to 8 for each). The results were normalized by arbitrarily setting the densitometry of untreated cells to 1.0. *P<0.05 vs control. †P<0.05 vs 100 nmol/L aldosterone. B, Effects of actinomycin D (AD; 5 μg/mL), cycloheximide (CH; 10 μg/mL), and PD98059 (PD; 100 μmol/L) on aldosterone-induced ERK1/2 activation in RMCs. Neither actinomycin D nor cycloheximide affect aldosterone-induced ERK1/2 phosphorylation at 10 minutes (n=4 to 6 for each). On the other hand, PD98059 prevents aldosterone-induced ERK1/2 phosphorylation (n=4). V indicates vehicle (0.01% ethanol).

Figure 5. A, Effects of eplerenone (10 μmol/L) and PD98059 (100 μmol/L) on aldosterone-induced cell proliferation, evaluated by [3H]-thymidine incorporation. Aldosterone (100 nmol/L) treatment for 30 hours significantly increases [3H]-thymidine incorporation. The aldosterone-induced increases in [3H]-thymidine incorporation are significantly inhibited by preincubation with eplerenone or PD98059 (n=8, respectively). B, Effects of eplerenone and PD98059 on aldosterone-induced alterations in the micromechanical properties, determined by a nanoidentation technique. Aldosterone treatment for 30 hours markedly decreases the elastic modulus (n=10), and these changes are abolished by preincubation with eplerenone or PD98059 (n=10, respectively). *P<0.05 vs control (vehicle 0.01% ethanol).

Figure 4.

Figure 5.

These data are inconsistent with the results of Rossol-Haseroh et al, who showed that the effects of aldosterone on ERK1/2 activity were insensitive to the classical MR antagonists, spironolactone, canrenoic acid, RU26752, and RU28318 in cortical-collecting duct cells. At present, we can find no satisfactory explanation for the discrepancy between our results and those of Rossol-Haseroh et al. However, it may be because of differences in the experimental conditions or cell types. Alternatively, it may be because of the specificity of the MR antagonists, as suggested by other investigators. In this regard, recent studies have indicated that part of the rapid actions of estrogen and other steroid hormones, including aldosterone, are mediated by intracellular steroid receptors. For example, Mazak et al showed that aldosterone potentiated Ang.
II–induced rapid ERK1/2 activation in vascular smooth muscle cells, and that this effect was abolished by treatment with spironolactone. Similarly, aldosterone-induced rapid activation of Ki-RasA, an activator of the ERK1/2 cascade, was markedly attenuated by spironolactone. 13 In addition, MR antagonists are able to block several non-genomic actions of aldosterone on vascular Na+, K+-ATPase, 14 arteriolar tone, 35,36 and Src kinase activation. 37 Based on these observations 13,28,29,32–37 along with the results of the present study, we speculate that in addition to the role as a transcription factor, MR could be involved in a cell signaling system involving the ERK1/2 pathway in RMCs, at least in part.

Perspectives
Recent clinical studies have reported the therapeutic potential of MR blockade for renal disease. 7–11 The present study provides evidence, for the first time, that MR is actually expressed in RMCs and involved in aldosterone-induced RMC injury, indicating that the glomerular mesangium is a major target for aldosterone and MR. These data may help to explain the recent clinical observations indicating blood pressure–independent renoprotective effects of MR antagonists. 7–11 In this study, involvement of ERK1/2 in the pathogenesis of aldosterone/MR-induced cellular injury was also indicated. Further studies are currently under way to elucidate the precise molecular mechanisms by which aldosterone mediates glomerular cell injury via activation of MR. In addition, determining how MR expression is regulated during the development of glomerular injury may lead to a better understanding of the pathophysiology of aldosterone-dependent renal injury.

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