Aldosterone Stimulates Collagen Gene Expression and Synthesis Via Activation of ERK1/2 in Rat Renal Fibroblasts

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Abstract—Recently, we demonstrated that in rats treated chronically with aldosterone and salt, severe tubulointerstitial fibrosis is associated with the activation of mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinases (ERK1/2). Here, we investigated whether aldosterone stimulates collagen synthesis via ERK1/2-dependent pathways in cultured rat renal fibroblasts. Gene expression of mineralocorticoid receptor (MR) and types I, II, III, and IV collagen was measured by real-time polymerase chain reaction (PCR). MR protein expression and ERK1/2 activity were evaluated by Western blotting analysis with anti-MR and anti–phospho-ERK1/2 antibodies, respectively. Collagen synthesis was determined by [3H]-proline incorporation. Significant levels of MR mRNA and protein expression were observed in rat renal fibroblasts. Treatment with aldosterone (0.1 to 10 nmol/L) increased ERK1/2 phosphorylation in a concentration-dependent manner with a peak at 5 minutes. Aldosterone (10 nmol/L) also increased the mRNA levels of types I, III, and IV collagen at 36 hours but had no effect on the type II collagen mRNA level. [3H]-proline incorporation was significantly increased by aldosterone in both the medium and cell layer at 48 hours. Aldosterone-induced ERK1/2 phosphorylation was markedly attenuated by pretreatment with eplerenone (10 μmol/L), a selective MR antagonist, or PD98059 (10 μmol/L), a specific inhibitor of MAPK kinase/ERK kinase, which is the upstream activator of ERK1/2. In addition, both eplerenone and PD98059 prevented the aldosterone-induced increases in types I, III, and IV collagen mRNA and [3H]-proline incorporation. These results suggest that aldosterone stimulates collagen gene expression and synthesis via MR-mediated ERK1/2 activation in renal fibroblasts, which may contribute to the progression of aldosterone-induced tubulointerstitial fibrosis. (Hypertension. 2005;46[part 2]:1039-1045.)

Key Words: aldosterone ■ collagen ■ fibroblasts ■ mineralocorticoids

Recent studies have indicated the usefulness of mineralocorticoid receptor (MR) antagonists in ameliorating renal injury.1–13 In stroke-prone spontaneously hypertensive rats4 and rats treated with angiotensin II and a nitric oxide synthase inhibitor,5 cyclosporine A,6 or radiation,7 MR antagonists had no effect on systemic blood pressure but markedly ameliorated glomerular and tubulointerstitial fibrosis. In clinical studies, the addition of a nonselective MR antagonist, spironolactone, to angiotensin-converting enzyme inhibitors had no hemodynamic effects but markedly reduced proteinuria in patients with chronic renal failure8 and early diabetic nephropathy.9 It has also been shown that monotherapy with spironolactone10 or eplerenone,11 a selective MR antagonist, is more effective than angiotensin-converting enzyme inhibitors in reducing proteinuria in hypertensive patients. Furthermore, White et al12 showed that in hypertensive patients, eplerenone had a similar blood pressure–lowering effect to that of a calcium antagonist, amlodipine, but reduced the urinary albumin-to-creatinine ratio to a greater extent. These observations suggest that MR blockade has renoprotective effects through mechanisms that cannot be simply explained by blood pressure and hemodynamic changes.

Recently, we reported that in rats, chronic treatment with aldosterone and salt resulted in severe tubulointerstitial fibrosis with an increased renal collagen content, and that these effects of aldosterone were prevented by concurrent treatment with eplerenone.13 Interestingly, we also found that tubulointerstitial injury and collagen accumulation were associated with the activation of mitogen-activated protein kinases, including extracellular signal-regulated kinases (ERK1/2).13 These data suggest that aldosterone/MR contributes to the pathogenesis of tubulointerstitial fibrosis through ERK1/2-dependent pathways.
In the present study, we hypothesized that aldosterone/MR may stimulate collagen synthesis through the activation of ERK1/2 in renal fibroblasts. To test this hypothesis, we first investigated whether MR is expressed in cultured rat fibroblasts. Next, we examined the effects of aldosterone on ERK1/2 phosphorylation, as well as collagen gene expression and synthesis in the renal fibroblasts. Finally, we examined the effects of eplerenone and PD98059, a specific inhibitor of mitogen-activated protein kinase/ERK kinase, which is the upstream activator of ERK1/2, on the aldosterone-induced collagen gene expression and synthesis.

Materials and Methods

Cell Culture
Rat renal fibroblasts (NRK-49F) were purchased from Dainippon Pharmaceutical Co Ltd and maintained according to published methods.\textsuperscript{15} Control solutions always contained an appropriate amount (≤1:1000 dilution for each) of vehicle: ethanol for aldosterone (Across Organics), DMSO for eplerenone (Pfizer Inc) and PD98059 (Merck KGaA), and distilled water for cycloheximide (Sigma Chemical Co). At subconfluence, the culture medium was replaced with serum-free medium for 24 to 30 hours to render the cells quiescent. After stimulation, protein or mRNA expression and synthesis.

Western Blotting Analysis
MR protein expression was determined by Western blotting analysis with an MR-specific antibody (Santa Cruz Biotechnology), as previously described in detail.\textsuperscript{16,17} To evaluate activated ERK1/2, immunoblotting was performed with an antibody against phospho-ERK1/2 (Cell Signaling Technology), as previously described.\textsuperscript{13,16,17} Total ERK1/2 protein expression was measured using a pan-ERK1/2 antibody (Cell Signaling Technology).\textsuperscript{13,16,17} All values were normalized by arbitrarily setting the densitometry of control samples to 1.0.

Polymerase Chain Reaction
The mRNA expression levels of MR, collagen types I, II, III, and IV and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were analyzed by real-time polymerase chain reaction (PCR), as described previously.\textsuperscript{13,17} Briefly, cDNA was initially denatured at 95°C for 30 seconds, and then amplified by PCR for 40 cycles (95°C for 15 seconds, 60°C for 40 seconds). The primer sequences are summarized in the Table. All data are expressed as the relative differences between control and treated cells after normalization to the GAPDH expression. The PCR product for MR (190 bp) was also electrophoresed in 2% agarose gels in Tris-borate EDTA buffer and then stained with ethidium bromide.

Proline Incorporation
[\textsuperscript{1}H]-proline incorporation was used for a marker of collagen synthesis.\textsuperscript{19–23} The levels of radiolabeled soluble collagen in the conditioned medium and nonsoluble collagen in the cell layer were determined.\textsuperscript{19,20}

Statistical Analysis
Values are presented as the mean±SE. One-way analysis of variance was used to determine the significance of differences among groups, after which a modified t test with the Bonferroni correction was used for comparisons between individual groups. \( P < 0.05 \) was considered statistically significant.

### Results

**Expression of MR in Rat Renal Fibroblasts**
Western blotting analysis with an MR-specific antibody yielded a prominent band of ≈110 kDa in lysates obtained from rat renal fibroblasts (Figure 1A). When immunoblotting was performed in the presence of the primary antibody and the peptide fragment of MR was used to generate the primary antibody (Santa Cruz Biotechnology), the observed band was displaced, confirming that the binding was specific for MR (Figure 1A).

As shown in Figure 1B, reverse-transcription PCR analysis revealed significant expression of the MR product (190 bp) in renal fibroblasts (n=5). Treatment with aldosterone (10 nmol/L) for 6 to 48 hours did not affect the MR mRNA level in renal fibroblasts (n=6 for each time point, Figure 1C).

**ERK1/2 Phosphorylation**
Aldosterone-induced (10 nmol/L) phosphorylation of ERK1/2 peaked at 5 minutes (2.0±0.2-fold) and continued for 30 minutes (n=7 to 9 for each time point) (Figure 2A). Figure 2B shows the concentration-dependent effects of aldosterone treatment (5 minutes) on the ERK1/2 phosphorylation. Aldosterone-stimulated ERK1/2 phosphorylation was found to be maximal at 10 nmol/L (n=7 to 10 for each concentration). However, no significant differences in the amounts of total (phosphorylated and unphosphorylated) ERK1/2 were observed in samples by Western blotting analysis with an anti-pan-ERK1/2 antibody (Figure 2A and 2B).

To investigate the contribution of MR to the aldosterone-induced ERK1/2 activation, the effects of a selective MR antagonist, eplerenone,\textsuperscript{1} on ERK1/2 phosphorylation were examined. Renal fibroblasts were pretreated with eplerenone (10 μmol/L) for 60 minutes before stimulation with aldosterone (10 nmol/L). Treatment with eplerenone prevented the aldosterone-induced ERK1/2 phosphorylation at 5 minutes (n=6) (Figure 3). To investigate the role of protein synthesis in the aldosterone-induced stimulation of ERK1/2, renal fibroblasts were pre-incubated with cycloheximide (10 μg/mL) for 15

### Primer Sequences and Product Size

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<th>Gene</th>
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<th>Product Size (bp)</th>
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minutes. However, cycloheximide had no effect on the aldosterone-induced ERK1/2 phosphorylation at 5 minutes (n = 6) (Figure 3). However, pre-incubation with a mitogen-activated protein kinase/ERK kinase inhibitor, PD98059 (10 μmol/L for 30 minutes), prevented the aldosterone-induced ERK1/2 phosphorylation (n = 6). The concentrations and pre-incubation times of eplerenone, cycloheximide, and PD98059 were determined on the basis of results from previous in vitro studies.16,21,24,25

Collagen Gene Expression

Figure 4 shows the effects of aldosterone on the mRNA levels of types I, II, III, and IV collagen (n = 7 to 9 for each). Treatment with aldosterone (10 nmol/L) significantly increased the mRNA levels of types I (175 ± 14% of the control), III (177 ± 9% of the control), and IV (261 ± 30% of the control) collagen, with peaks at 36 hours, but had no effect on the type II collagen mRNA level. Pretreatment with eplerenone (10 μmol/L) or PD98059 (10 μmol/L) prevented the aldosterone-induced increases in the mRNA levels of types I, III, and IV collagen (Figure 5) (n = 9 for each).

Collagen Synthesis

The effects of aldosterone on collagen synthesis were evaluated by [3H]-proline incorporation in rat renal fibroblasts (n = 11 for each) (Figure 6). Treatment with aldosterone (10 nmol/L) for 48 hours significantly increased [3H]-proline incorporation in the conditioned medium (135 ± 11% of the control) and cell layer (135 ± 14% of the control). These aldosterone-induced increases in [3H]-proline incorporation were inhibited by pre-incubation with eplerenone or PD98059.
Discussion

Aldosterone has been reported to induce collagen synthesis in cultured cardiac fibroblasts, vascular smooth muscle cells, and glomerular mesangial cells. However, to the best of our knowledge, there has been no direct evidence showing that aldosterone directly stimulates collagen gene expression and/or synthesis in renal fibroblasts. The present study demonstrated that cultured rat renal fibroblasts express both MR mRNA and protein. The results further revealed that aldosterone directly stimulates collagen gene expression and synthesis in renal fibroblasts. In addition, the effects of aldosterone were prevented by pretreatment with the selective MR antagonist, eplerenone. Taken together, these data support the hypothesis that aldosterone/MR plays an important role in the pathophysiology of collagen accumulation in renal fibroblasts.

In the present study, we measured [3H]-proline incorporation as an index of collagen synthesis, because it has been shown that both labeled soluble proteins secreted into the medium and cell-associated proteins reflect the newly synthesized collagen. However, this method does not provide any information on the individual collagen types involved. Several clinical studies have reported that interstitial mRNA expression of fiber-forming collagens (collagen types I and III) and collagen type IV, which is usually situated in cell basement membranes, is significantly increased in patients with renal fibrosis. Interestingly, it was also demonstrated that the increased amounts of renal interstitial collagens were co-localized with fibroblasts. These findings suggest that fibroblasts play a role in renal fibrosis through the synthesis of types I, III, and IV collagen. In the present study, aldosterone...
increased the gene expression of collagen types I, III, and IV in renal fibroblasts. However, the specific contributions of these aldosterone-induced collagens gene expression to the pathophysiology of renal fibrosis remain unclear, and further studies are necessary to address these issues.

ERK1/2 is an important mediator of the intracellular signal transduction pathway responsible for collagen synthesis in cardiovascular tissues, including the kidney. Partially, Pat et al. revealed that tubulointerstitial fibrosis induced by unilateral ureteral obstruction was associated with ERK1/2 phosphorylation in rats. Interestingly, unilateral ureteral obstruction–induced tubulointerstitial fibrosis was reported to be markedly attenuated by treatment with spironolactone, suggesting that aldosterone/MR may contribute to its pathogenesis. Recently, we showed that aldosterone-induced tubulointerstitial fibrosis and collagen accumulation were associated with the activation of ERK1/2 in rats. Furthermore, the aldosterone-induced tubulointerstitial fibrosis and ERK1/2 activation were prevented by concurrent treatment with spironolactone, suggesting that aldosterone/MR induces tubulointerstitial fibrosis through the activation of ERK1/2. In the present study, aldosterone-induced collagen gene expression and synthesis were prevented by pretreatment with eplerenone or the mitogen-activated protein kinase/ERK kinase inhibitor, PD98059. These data combined with those of the previous animal experiments indicate that ERK1/2 is an important mediator of aldosterone-induced collagen synthesis in renal fibroblasts, which may contribute to the pathogenesis of aldosterone/MR-induced tubulointerstitial fibrosis.

In addition to its classical genomic actions mediated through regulation of nuclear gene transcription and protein synthesis, aldosterone also elicits rapid, potentially nongenomic responses in a variety of cells. Therefore, we examined the effects of cycloheximide, an inhibitor of protein synthesis, on the aldosterone-induced ERK1/2 phosphorylation. The results revealed that cycloheximide had no effect on the rapid aldosterone-induced phosphorylation of ERK1/2, suggesting that nongenomic mechanisms are involved in aldosterone-induced ERK1/2 activation. We also observed that pre-incubation with eplerenone significantly attenuated the rapid effect of aldosterone on ERK1/2 in rat fibroblasts. These observations are consistent with those of recent studies showing that MR antagonists block aldosterone-induced activation of ERK1/2 in mesangial cells and Chinese hamster ovary cells transfected with human MR. Similarly, MR antagonists prevented aldosterone-induced p38 mitogen-activated protein kinase activation and angiotensin II–induced ERK1/2 activation in vascular smooth muscle

**Figure 5.** Effects of eplerenone and PD98059 on aldosterone-induced collagen mRNA expression in rat renal fibroblasts. Pretreatment with eplerenone or PD98059 prevents the aldosterone-induced increases in the mRNA levels of types I, III, and IV collagen. All data are expressed as the relative differences after normalization to the GAPDH expression. *P<0.05 vs vehicle.

**Figure 6.** Effects of eplerenone and PD98059 on aldosterone-induced collagen synthesis, evaluated by [3H]-proline incorporation. Aldosterone treatment significantly increases [3H]-proline incorporation in the conditioned medium and cell layer. These aldosterone-induced increases in [3H]-proline incorporation are inhibited by pre-incubation with eplerenone or PD98059. *P<0.05 vs vehicle.
cells. However, Rossol-Haseroth et al\textsuperscript{25} reported that the effects of aldosterone on ERK1/2 phosphorylation were unaffected by MR antagonists in cortical collecting duct cells. At present, we can find no satisfactory explanation for the discrepancies among these results. However, they may arise because of differences in the experimental conditions or cell types. Spironolactone has been shown to markedly attenuate the rapid aldosterone-induced activation of Ki-RasA\textsuperscript{30} and c-Src,\textsuperscript{21} activators of the ERK1/2 cascade. In addition, MR antagonists are able to block several nongenomic actions of aldosterone on vascular Na\textsuperscript{+}K\textsuperscript{+}-ATPase\textsuperscript{34} and arterial.\textsuperscript{35,36} Based on these observations,\textsuperscript{16,21,24,31–36} together with the results of the present study, we speculate that in addition to its role as a transcription factor, MR could be involved, at least partially, in a cell signaling system involving the ERK1/2 pathway in rat renal fibroblasts.

**Perspectives**

The present study provides evidence, for the first time to our knowledge, that MR is actually expressed in renal fibroblasts, and that it is involved in aldosterone-induced collagen gene expression and synthesis. These data indicate that renal fibroblasts are one of the major targets for aldosterone/MR. In addition, these data may help to explain recent clinical observations indicating that MR antagonists have blood pressure-independent renoprotective effects.\textsuperscript{8–12} The results of the present study further suggest that ERK1/2 is an important mediator of aldosterone/MR-induced collagen synthesis. Future studies are required to elucidate the precise molecular mechanisms by which aldosterone/MR mediates collagen synthesis in renal fibroblasts.

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