Osteopontin (OPN) is a glycosylated phosphoprotein that contains an arginine-glycine-aspartate (RGD) binding sequence that enables interaction with various integrins and CD44. OPN is produced by osteoblasts, macrophages, endothelial cells, and epithelial cells and acts to facilitate cell adhesion and migration. In the normal kidney, OPN is mainly expressed in the loop of Henle and distal nephron. OPN expression is upregulated in renal tubular cells and OPN is therefore of interest that we have recently reported the independent association of plasma OPN levels with serum aldosterone levels in patients with essential hypertension. In addition, plasma OPN levels were higher in patients with primary aldosteronism than with essential hypertension. One of the direct actions of aldosterone may be the induction of systemic fibrosis by increasing proinflammatory cytokines including OPN.

Previous studies have shown that various inducers regulate OPN expression at the transcriptional level with transcription factors such as Sp-1, activator protein 1 (AP-1),\textsuperscript{9,10} and nuclear factor kappa B (NF\textsubscript{κB}). However, the transcriptional regulatory mechanism of OPN by aldosterone remains unclear. In the present study, we demonstrated that aldosterone induces the transcription of the OPN gene through AP-1 and NF\textsubscript{κB} via MR and plays an important role in renal fibrosis via aldosterone.

**Abstract**—Osteopontin (OPN), a proinflammatory cytokine, plays an important role in renal fibrosis. We reported that plasma OPN levels were higher in patients with primary aldosteronism than with essential hypertension. However, the regulatory mechanism of OPN by aldosterone remains unclear. Here, we report the transcriptional regulation of OPN expression by aldosterone and the functional effects of aldosterone-mediated OPN transcription in renal fibroblasts. Aldosterone induced OPN expression in a dose-dependent manner with significant responses at 10 nmol/L (1.6±0.2-fold of controls, \(P<0.05\), \(n=5\)) and elicited maximal effects at 10 μmol/L (3.5±0.4-fold of controls, \(P<0.01\), \(n=5\)). Aldosterone increased OPN expression in a time-dependent manner with a maximal effect after 48 hours (2.7±0.3-fold of controls, \(P<0.01\), \(n=5\)). This effect was abolished by the mineralocorticoid receptor (MR) antagonist spironolactone.

Luciferase promoter deletion assays identified a novel cis regulatory element (−2153 to −1758) in the OPN promoter that is responsive to aldosterone. This element contains an activator protein-1 (AP-1) and nuclear factor kappa B (NF\textsubscript{κB}) site. Electrophoretic mobility shift assays, supershift assays, and chromatin immunoprecipitation assays identified both AP-1 and NF\textsubscript{κB} as the DNA binding proteins induced by aldosterone with spironolactone inhibiting aldosterone-induced AP-1 or NF\textsubscript{κB} activity. OPN-siRNA inhibited completely the induction of cell proliferation, type I, III, and IV collagen synthesis by aldosterone. These results indicate that aldosterone induced MR-mediated OPN expression through AP-1 and NF\textsubscript{κB} activation and suggest that aldosterone plays an important role in renal fibrosis through the induction of OPN. (Hypertension. 2008;51[part 2]:507-513.)

**Key Words:** osteopontin • aldosterone • activator protein-1 • nuclear factor kappa B • mineralocorticoid receptor • renal fibrosis
A chromatin immunoprecipitation (ChIP) assay was performed using ChIP assay kit (Upstate) according to the manufacturer’s instructions. Soluble chromatin was prepared from renal fibroblasts treated with 10 μmol/L spironolactone for 1 hour followed by stimulation with aldosterone (100 ng/mL) for 30 minutes. Chromatin was immunoprecipitated with the indicated antibodies (2 μg each). The extracted DNAs were polymerase chain reaction (PCR) amplified using primer pairs that cover AP-1 sequence in the OPN promoter as follows: forward 5'- GTTGAGTCATTCCTGTGGGC-3' and antisense 5'-CACCAGCTAGCATAATG-3'. The PCR products were separated by electrophoresis on a 2.5% agarose gel.

Statistical Analysis
Significance was determined using unpaired Student t test, Mann-Whitney U test, or ANOVA, followed by Tukey test. All data are expressed as mean±SD, and statistical significance was defined as P<0.05.

Results
Induction of OPN Expression in Renal Fibroblasts by Aldosterone
We first examined whether aldosterone induces OPN gene expression in renal fibroblasts. As shown in Figure 1A, aldosterone (100 nmol/L) significantly increased steady-state OPN mRNA levels in a time-dependent manner (3 to 48 hours). A significant increase was observed at 3 hours (1.8±0.2-fold, P<0.05, n=5) with peak expression observed at 48 hours (2.7±0.3-fold, P<0.01, n=5). In addition, aldosterone dose-dependently increased steady-state OPN mRNA levels in renal fibroblasts (Figure 1B). A 1.6±0.2-fold increase was induced by aldosterone doses as low as 10 nmol/L (P<0.05, n=5) with a 3.5±0.4-fold increase evident at 10 μmol/L (P<0.01, n=5). These results suggested that aldosterone regulation of OPN expression by renal fibroblasts occurred at the level of gene expression.

OPN Expression Induced by Aldosterone Is via MR
We used a selective MR antagonist spironolactone to determine whether aldosterone-induced OPN expression was mediated via the MR. The induction of OPN mRNA expression by aldosterone (100 nmol/L) was completely blocked by pretreatment with spironolactone (10 μmol/L) thereby indicating that aldosterone-induced OPN gene expression in renal fibroblasts is mediated via the MR (Figure 1C). In addition, the aldosterone-induced OPN gene expression was completely inhibited by pretreatment with a transcription inhibitor, actinomycin D (5 μmol/L), indicating its transcription-dependent action (Figure 1C).

Identification of cis-Regulatory Element in the OPN Promoter
To identify the cis-regulating elements of the OPN promoter that regulated OPN gene transcription induced by aldosterone in renal fibroblasts, we constructed a set of pGL4 plasmids carrying 5' deletions of the OPN promoter (Figure 2A). Luciferase reporter activity was determined in renal fibroblasts transiently transfected with these deleted constructs and then stimulated by aldosterone (100 nmol/L) for 24 hours. Luciferase activity of Luc-2153 (2.3±0.2-fold, P<0.001, n=24) and Luc-1839 (2.6±0.3-fold, P<0.001, n=24) in aldosterone-stimulated cells was higher than unstimulated cells, whereas Luc-1758 to Luc-174 led to the loss of luciferase activity by aldosterone stimulation. These results suggested that a cis-regulating element responding to aldosterone was located in the −2153 to −1758 region of the distal OPN promoter.
Effect of Point Mutagenesis of the AP-1 or NFκB Binding Motif on Promoter Activity

We searched for transcription factor binding sites between -11002 to 1758 with TFSEACH (http://mbs.cbrc.jp/research/db/TFSEACH.html). This region contained an AP-1 binding site at -1870 and a NFκB binding site at -1800. Both the AP-1 and the NFκB binding site mutation abolished the activation of promoter activity by aldosterone (Figure 2C).

Electrophoretic Mobility Shift Assay Analysis

With the AP-1 and NFκB Binding Site in the −2153 to −1758 Region of the OPN Promoter

When nuclear extracts were incubated in the presence of the 32P-labeled probe 1 (containing AP-1 potential site), the complex density peaked at 1 hour in the nuclear extracts of cells treated by aldosterone and remained elevated for 3 hours (Figure 3A). After the addition of an excess of cold probe 1, only the upper complex disappeared, thereby indicating that the lower complex was a nonspecific band. Incubation of nuclear extracts from aldosterone-stimulated cells with the 32P-labeled probe 2 (containing NFκB potential site) resulted in complexes being detected by electrophoretic mobility shift assay (EMSA) (Figure 3B). Binding of factors to the NFκB site was maximal at 30 minutes of treatment with aldosterone. These complexes disappeared when an excess of the specific cold probe 2 was added but not after the addition of an excess of nonspecific cold probe. We next examined the effect of spironolactone on the formation of protein/DNA complexes at each AP-1 or NFκB binding site on the OPN promoter sequence (Figure 3A and 3B). Spironolactone (10 μmol/L) inhibited aldosterone-induced AP-1 and NFκB binding activities. These findings indicated that aldosterone induces AP-1 and NFκB activity through the MR.

The AP-1 Site Activity by c-Fos and c-Jun and the NFκB Site Activity by p65 of the OPN Promoter

We next performed supershift experiments using anti–c-Fos and c-Jun antibody (Figure 4A). Antibody against c-Fos substantially diminished complex formation and supershifted complexes using probe 1 in the OPN promoter. In addition, anti–c-Jun antibody partially supershifted the AP-1 complex, whereas anti-NFκB antibodies to p50 and p65 had no effect on the AP-1 complex formation. This experiment showed that the transcription factors c-Fos and c-Jun are both components of the aldosterone-activated complex.

Supershift assays using anti-p50 or -p65 anti-NFκB antibodies were performed to identify the transcription factors binding the NFκB site (Figure 4B). When anti-p65 antibody was added to nuclear extracts isolated from aldosterone-stimulated cells, a diminished and supershifted complex was observed but this was not evident after the addition of anti-p50 antibody. In contrast, no modification was observed when an anti–c-Fos and c-Jun antibody was used. These
results demonstrate that p65 NFκB binds the site located at −1800 in nuclear extracts.

To confirm that c-Fos, c-Jun, and p65 bind to the endogenous OPN promoter we next performed ChIP assays (Figure 4C). ChIP assays demonstrated that this OPN promoter region coimmunoprecipitated with c-Fos, c-Jun, and p65 after stimulation with aldosterone. These inductions were inhibited by spironolactone.

Role of AP-1 and NFκB in the Aldosterone-Induced OPN Expression

To evaluate the role of AP-1 in the induction of OPN mRNA expression by aldosterone, we used a phosphorothioate-modified oligodeoxynucleotide (ODN) containing the specific sequence for AP-1 or NFκB on the OPN promoter. The decoy ODNs were added to renal fibroblasts 6 hours before aldosterone stimulation. As shown in Figure 5, each decoy

Figure 3. A, Probe 1 containing the AP-1 potential site was used for the EMSA with nuclear extracts from quiescent renal fibroblasts stimulated by 100 nmol/L aldosterone for the indicated times. Competition experiments were performed using 100-fold excess of unlabeled oligonucleotides (S, specific probe; NS, nonspecific probe). Spironolactone suppresses AP-1 binding activity. After pretreatment with spironolactone (10 μmol/L) for 60 minutes, renal fibroblasts were stimulated with 100 nmol/L aldosterone for 60 minutes. B, Probe 2 containing the NFκB potential site was used for the EMSA with nuclear extracts from quiescent renal fibroblasts stimulated with 100 nmol/L aldosterone for the indicated times. Spironolactone suppresses NFκB binding activity. After pretreatment with spironolactone (10 μmol/L) for 60 minutes, renal fibroblasts were stimulated with 100 nmol/L aldosterone for 30 minutes. These figures present 1 representative experiment of 3.

Figure 4. A and B, Characterization of the AP-1 or NFκB complex was performed by supershift assays. Serum-deprived renal fibroblasts were stimulated with aldosterone and 5 μg nuclear extracts were incubated with the indicated antibodies (0.5 μg) before the addition of the radiolabeled probe. Figures shown are representative of 3 independently performed experiments. C, ChIP assays were performed using chromatin isolated from serum-deprived renal fibroblasts pretreated for 1 hour with vehicle or 10 μmol/L spironolactone and stimulated with aldosterone (100 nmol/L) for 30 minutes. Rabbit IgG or c-Fos, c-Jun, and p65 antibodies were used for immunoprecipitation. Total extract (Input) was used as positive PCR control. Autoradiograms shown are representative of 3 independently performed experiments.
ODN inhibited aldosterone-induced OPN mRNA expression. These results strongly indicated that aldosterone-induced OPN expression involves both AP-1 and NFκB.

Inhibitory Effect of OPN Small Interfering RNA on the Stimulation of Renal Fibroblast Cell Proliferation and Collagen Gene Expression by Aldosterone

To assess the role of the upregulated OPN expression by aldosterone, we analyzed cell proliferation and collagen synthesis in OPN knockdown cells treated by RNA interference. To confirm the effectiveness of OPN-small interfering RNA (siRNA), we performed immunoblot analysis and determined the OPN expression level (Figure 6A). Compared with control siRNA-treated cells, OPN expression was suppressed significantly with almost negligible expression evident in OPN siRNA-treated cells. Furthermore, aldosterone treatment significantly increased the induction of cell proliferation (145±7% of controls, P<0.05, n=60; Figure 6B) and collagens type I (155±13% of controls, P<0.05, n=5), III (170±14% of controls, P<0.01, n=5), and IV (163±20% of controls, P<0.01, n=5) mRNA synthesis (Figure 6C). Aldosterone-induced increases in cell proliferation and collagen I, III, and IV collagen expression were significantly inhibited by OPN siRNA transfection (105±6% of controls, 102±11% of controls, 58±10% of controls, 103±12% of controls, respectively, n=5). These results suggested that aldosterone-induced OPN expression plays an important role in renal fibrosis.

Discussion

An accumulating body of evidence indicates that aldosterone is an important mediator of renal injury and vascular inflammation and acts by inducing a variety of proinflammatory genes (including OPN, intercellular adhesion molecule-1 [ICAM-1], vascular cell adhesion molecule [VCAM]-1, and monocyte chemoattractant protein-1) independent of its blood pressure-elevating effect. Aldosterone is a steroid hormone that binds to the MR, a ligand-activated transcription factor. Steroid hormone receptors bind to specific DNA sequences in the promoters of hormone-responsive genes and recruit cofactors in a ligand-dependent manner, thereby modulating gene expression. MR binds in vitro to glucocorticoid response elements (GRE) and mineralocorticoid response elements (MRE) in a ligand-dependent manner. Although distinct MRE has not been identified, recently Gauer et al reported aldosterone-mediated induction of OPN via the MR and identified a putative MRE that was located 1984 bp upstream of the OPN coding sequence in rat mesangial cells. Because this MRE is located in a novel cis...
element which was identified in this study, we investigated the direct interaction between MR and this MRE on the OPN promoter after aldosterone stimulation. Renal fibroblasts were incubated for 1 to 4 hours with various doses of aldosterone (1 nmol/L to 10 μmol/L), and nuclear extracts were incubated with labeled MRE oligodeoxynucleotides. Although DNA-protein complexes were analyzed by EMSA, we were unable to detect the specific signal of the complex (data not shown). We do not have a satisfactory explanation for this discrepancy. However, it might be secondary to differences in the experimental conditions or cell types. Because the OPN promoter does not have another putative MRE and GRE, aldosterone-induced OPN transcription in renal fibroblasts is mediated by an indirect mechanism through regulation of other transcription factors such as AP-1 and NFκB as shown in this study. Li et al reported that aldosterone increased NFκB activity and NFκB target gene-tumor necrosis factor (TNF)-α gene expression in hepatic stellate cells (HSCs) T6 cells. Aldosterone also increased AP-1 activity and α 1 protocollagen gene expression, an AP-1 target gene, in HSC-T6 cells.13 They concluded that aldosterone mediates hepatic fibrogenesis via stimulation of the NFκB and AP-1 pathway. Although they did not mention OPN expression in their study, their work and our study indicate that aldosterone stimulates the transcriptional factors NFκB and AP-1 and can induce organ fibrosis.

A complete loss of inducible OPN promoter activity after each point mutagenesis of AP-1 or NFκB site strongly suggested an interaction between AP-1 and NFκB as well as the importance of these sites on transcriptional activity of the OPN promoter. Moreover, NFκB has been reported to interact with some transcription factors including AP-1.16 However, in the present study, no modification was observed when anti–c-Fos or c-Jun and anti-p65 antibody was added to the nuclear extract with probe 2 and probe 1 in the EMSA, respectively. These results suggest that NFκB and AP-1 independently contribute to the induction of OPN during aldosterone stimulation in renal fibroblasts. However further study is needed to clarify the interaction between AP-1 and NFκB on stimulation of OPN gene transcription.

Previously, the AP-1 binding site −1870 and the NFκB binding site −1800 in the rat OPN promoter has been reported to be the cis elements which activate OPN promoter activity by uridine triphosphate (UTP) in smooth muscle cells.9,17 In the AP-1 binding site −1870, UTP induces AP-1 activation by both c-Fos and cAMP responsive element-binding protein (CREB) binding. We performed the supershift assay using anti-CREB antibody and the AP-1 complex was not influenced, thereby indicating that CREB is not involved in the transcriptional regulation of OPN by aldosterone in renal fibroblasts (data not shown). The signal transduction system of OPN gene transcription by UTP is similar but not identical to that of aldosterone.

We demonstrated, using EMSA and ChIP assay experiments, that spironolactone suppresses AP-1 and NFκB binding to each response element in the OPN promoter suggesting that AP-1 and NFκB binding and activation is initiated through the MR in renal fibroblasts. Nishiyama et al reported that eplerenone, a selective MR antagonist, attenuated the rapid action of aldosterone on extracellular signal regulated kinase (ERK) 1/2 phosphorylation in rat mesangial cells. Because this rapid ERK1/2 phosphorylation for 10 minutes was not influenced by either actinomycin D or cycloheximide, they proposed that this phenomenon was a nongenomic effect and mediated through the MR.18 NFκB can be involved in rapid transcription without new protein synthesis. In the present study, aldosterone activated NFκB for 30 minutes suggesting that aldosterone transactivated NFκB by a nongenomic effect through the MR. In contrast, AP-1 activation requires transcription. Our study demonstrated that aldosterone activated AP-1 for 1 hour suggesting that aldosterone transactivates AP-1 by genomic action through the MR. However, additional studies are necessary to delineate in more detail the molecular mechanism involved in the regulation of c-Fos/c-Jun and p65 transactivation by aldosterone.

Downstream events that followed aldosterone-stimulated OPN expression are demonstrated in the present study. Nagai et al reported that aldosterone stimulated collagen gene expression, including collagens type I, III, and IV, and collagen synthesis via MR-mediated ERK1/2 activation in NRK-49F renal fibroblasts.11 Collagens type I and III are matrix proteins that are produced in excess during renal fibrosis. Previous studies have reported that not only collagens type I and III but also collagen type IV, a basal membrane protein, is increased in patients with renal fibrosis such as crescentic glomerulonephritis.19,20 Goumenos et al demonstrated that the site of synthesis of collagen III and IV appeared to be confined to fibroblasts in crescentic glomerulonephritis.20 These findings suggest that renal fibroblasts play an important role in renal fibrosis through the synthesis of collagen types I, III, and IV. In the present study, RNA interference of OPN expression significantly reduced cell proliferation and the gene expression of collagens type I, III, and IV. The present study therefore indicates that aldosterone-induced OPN expression plays a key role in aldosterone-induced renal fibrosis.

Perspectives
The present data demonstrated that aldosterone-induced OPN expression in renal fibroblasts depends on AP-1 and NFκB binding to the distal OPN promoter. Spironolactone inhibited aldosterone-induced OPN expression and reduced AP-1 and NFκB binding to the OPN promoter. OPN siRNA suppressed aldosterone-induced cell proliferation and collagen gene expression. Because OPN is a key component for the aldosterone-induced renal fibrosis, inhibition of OPN could provide a potential target for therapeutic intervention in renal disease.

Disclosures
None.

References


Osteopontin in Rat Renal Fibroblasts: Functional Properties and Transcriptional Regulation by Aldosterone

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