Aldosterone-Stimulated SGK1 Activity Mediates Profibrotic Signaling in the Mesangium

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ABSTRACT

Several recent reports support the hypothesis that aldosterone contributes to the progression of renal injury. Mineralocorticoids increase the expression of serum- and glucocorticoid-inducible protein kinase 1 (SGK1), which is upregulated in several fibrotic diseases. It was hypothesized that SGK1 may mediate the effects of aldosterone on glomerular fibrosis and inflammation. In primary cultures of rat mesangial cells, aldosterone stimulated the expression, phosphorylation, and kinase activity of SGK1, as well as SGK1-dependent NF-κB activity. Furthermore, aldosterone augmented the promoter activity and protein expression of intercellular adhesion molecule-1 (ICAM-1), which modulates the inflammatory response, and the profibrotic cytokine connective tissue growth factor (CTGF) in an SGK1- and NF-κB-dependent manner. Similar to the in vitro results, uninephrectomized rats that were treated with aldosterone demonstrated increased glomerular expression of SGK1, ICAM-1, and CTGF proteins than untreated rats; these changes were accompanied by hypertension, glomerulosclerosis, and inflammation. In conclusion, these findings suggest that aldosterone stimulates ICAM-1 and CTGF transcription via the activation of SGK1 and NF-κB, effects that may contribute to the progression of aldosterone-induced mesangial fibrosis and inflammation.


Accumulating evidence suggests that angiotensin-converting enzyme (ACE) inhibition or angiotensin II receptor (ATR) blockade attenuates the decline in renal function and structural damage in various kidney diseases.1–4 These benefits of ACE inhibition and ATR blockade are probably attributed to the suppression of intrarenal angiotensin II concentrations and the changes that follow as a consequence.4,5 Recent clinical and experimental studies have demonstrated that elevated plasma aldosterone may also contribute to the progression of cardiac and renal disease.6,7 Greene et al.8 demonstrated a significant suppression of hyperaldosteronism and a marked attenuation of proteinuria, hypertension, and glomerulosclerosis in a remnant kidney model using rats that were treated with enalapril and losartan. Similarly, Rocha et al.9 demonstrated the renoprotective effects of eplerenone and spironolactone in an aldosterone-stimulated rat model. Chrysostomou and Becker7 found that the addition of spironolactone to ACE inhibitors markedly reduced the urinary excretion rate of protein in patients with chronic renal failure without exerting hemodynamic effects. All of these studies strongly suggest that aldosterone is involved in the pathogenesis of renal injury.

Our group recently revealed that aldosterone stimulates the mitogen-activated protein kinase

Received May 2, 2007. Accepted September 6, 2007.
Published online ahead of print. Publication date available at www.jasn.org.

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pathway and cell-cycle progression in mesangial cells mainly via the mineralocorticoid receptor (MR).\textsuperscript{10} Gathering evidence implicates serum- and glucocorticoid-inducible protein kinase (SGK1) as an important actor in the regulation of salt reabsorption by mineralocorticoids.\textsuperscript{11,12} The sgk1 gene was originally cloned as a glucocorticoid-sensitive\textsuperscript{13} or a cell volume-regulated gene,\textsuperscript{14} then later was demonstrated to be strongly upregulated by mineralocorticoids.\textsuperscript{13–15} SGK1 is expressed in the collecting system and glomeruli of the kidney.\textsuperscript{16,17} SGK1 transcript levels have been reported to be elevated in several fibrotic diseases, including diabetic nephropathy,\textsuperscript{18} glomerulonephritis,\textsuperscript{19} lung fibrosis,\textsuperscript{20} and liver cirrhosis.\textsuperscript{21} Glomerular inflammation and fibrosis are the two major processes involved in the progression of glomerulosclerosis. In this study, we investigated the potential involvement of SGK1 in the aldosterone-induced expressions of connective tissue growth factor (CTGF) and intercellular adhesion molecule-1 (ICAM-1), a typical fibrosis-related gene and a typical inflammation-related gene, respectively, in rat mesangial cells. CTGF is a key mediator of matrix protein formation and is upregulated in several fibrotic renal diseases, including diabetic nephropathy and glomerulosclerosis.\textsuperscript{22–24} ICAM-1 was reported to be one of the most important adhesion molecules in the process of glomerular inflammation.\textsuperscript{25} Although expression of ICAM-1 is usually weak or absent in the glomeruli, ICAM-1 is upregulated in the mesangium and endothelial cells in many forms of human glomerulonephritis.\textsuperscript{26,27} We hypothesized that aldosterone can stimulate SGK1 activity and induce CTGF and ICAM-1 expressions, mainly via NF-κB in rat mesangial cells.

The purpose of this study was to investigate the mechanisms of aldosterone-induced SGK1 activation and the inflammatory and fibrotic signals in glomerular sclerosis. We attempted to determine the mechanisms behind the glomerular sclerosis of aldosterone by investigating the regulation of SGK1, the regulation of the NF-κB pathway, and the transcriptional regulation of CTGF and ICAM-1 both in vitro and in vivo. Our results indicated the following: Aldosterone stimulates SGK1 phosphorylation and protein expression in mesangial cells via MR; aldosterone stimulates NF-κB, at least in part, via the activation of SGK1; and aldosterone stimulates ICAM-1 and CTGF transcription via NF-κB and SGK1. MR antagonists may serve as therapeutic targets for the treatment of mesangial proliferative disease.

RESULTS

Specificity of Anti-SGK1 and Anti–phospho-SGK1 (Thr-256) Antibodies

We first examined the specificity of the anti-SGK1 antibody using wild-type, SGK1-overexpressing mesangial cells (positive control) and small interference RNA (siRNA)-transfected mesangial cells (negative control). This siRNA was functionally validated SGK1 siRNA (ID#50754; Ambion, Lafayette, CO). As shown in Figure 1A, the band at 48 kD in the mesangial cells was significantly enhanced by wild-type SGK1 transfection and dramatically reduced by transfection with siRNA. In contrast, control siRNA (scrambled sequence) had no effects on SGK1 expression. On the basis of these results, we were able to confirm the specificity of the anti-SGK1 antibody. We next examined the specificity of the anti–phospho-SGK1 (Thr-256) antibody. Mesangial cells were transfected with wild-type SGK1 or siRNA, incubated with a medium containing \textsuperscript{32}Porthophosphate, and then incubated with aldosterone (10 nM). After immunoprecipitation with anti-SGK1 antibody, the immune complex was analyzed by Western blotting with anti-SGK1 and anti–phospho-SGK1 (Thr-256) antibodies. We also exposed the membrane to x-ray film to detect \textsuperscript{32}P incorporation into the immune complex. Similar results were obtained from six replicate experiments.
poration after transfection with the wild-type SGK1 and significantly reduced incorporation after transfection with SGK1 siRNA. The similar changes in intensity of the 48-kD band, detected using the anti–phospho-SGK1 (Thr-256) antibody and ^32^P incorporation into the immune complex, confirmed that this phospho-specific antibody recognizes only the phosphorylated form of SGK1 (Thr-256). Similar results were obtained from six replicate experiments (Figure 1).

**Aldosterone Stimulates the Expression, Phosphorylation, and Kinase Activity of SGK1 in Rat Mesangial Cells via MR**

In this experiment, we sought to elucidate the signaling pathway of aldosterone in the mesangial cells by investigating the effects of aldosterone on the expression, phosphorylation, and kinase activity of SGK1. We began by examining whether aldosterone stimulates SGK1 expression and phosphorylation. As shown in Figure 2A, aldosterone stimulated the phosphorylation of SGK1 at Thr-256 from 3 h onward and increased the protein expression of SGK1 from 6 h. We also examined the time course of aldosterone-induced SGK1 protein expression in a human renal cortical epithelial (HRCE) cell line, in which SGK1 induction by aldosterone has previously been reported. The time course of SGK1 induction was earlier in the HRCE cell line than in rat mesangial cells (Figure 2A). The increments of SGK1 expression and phosphorylation of SGK1 were dosage dependent from 0.1 to 10 nM (data not shown). The aldosterone-induced increments of SGK1 phosphorylation and expression were inhibited by an MR inhibitor (eplerenone; Figure 2, C and D). Last, we found that aldosterone enhanced the SGK1 activity, whereas eplerenone reversed the enhancement (Figure 2E).

**Aldosterone Stimulates SGK1 Expression Partially via MEK1 in Mesangial Cells**

Next, we sought to determine the signaling pathway of aldosterone in relation to SGK1 expression by examining how MEK1 inhibitor (PD98051), p38K inhibitor (SB203580), and phosphatidylinositol-3’ kinase (PI3K) inhibitors (LY294002 and wortmannin) influenced the aldosterone-stimulated enhancement of SGK1 expression. As shown in Figure 3, A and B, the aldosterone-stimulated SGK1 expression was significantly attenuated by the MEK1 inhibitor but exhibited no significant change in response to the p38K inhibitor or the PI3K inhibitors. Mesangial cells transfected with siRNA for SGK1 were used as a negative control. To confirm the involvement of the MEK1 pathway in this SGK1 enhancement, we transfected dominant negative MEK1 and wild-type MEK1 into mesangial cells. As shown in Figure 3, C and D, the transfection with dominant negative MEK1 significantly reduced the SGK1 expression. We also examined the efficiency of transfection with MEK1 plasmids. As shown in Figure 3C, MEK1 expression was significantly enhanced by wild-type MEK1 transfection.

**Aldosterone Stimulates SGK1 Phosphorylation via PI3K in Mesangial Cells**

In this experiment, we investigated the effects of PI3K inhibitors (LY294002 and wortmannin) on aldosterone-stimulated SGK1 phosphorylation by determining the signaling pathway of aldo-
Aldosterone in relation to SGK1 phosphorylation and activation. As shown in Figure 4, A and B, the aldosterone-stimulated SGK1 phosphorylation was significantly attenuated by the PI3K inhibitors but showed no significant change in response to the MEK1 or p38K inhibitors. Mesangial cells transfected with siRNA for SGK1 were used as a negative control. To confirm the involvement of the PI3K pathway in the enhancement of SGK1 phosphorylation, we transfected dominant negative PI3K and wild-type PI3K adenoviruses into mesangial cells. As shown in Figure 4, C and D, the transfection with dominant negative PI3K significantly reduced the aldosterone-stimulated SGK1 phosphorylation.

**Aldosterone Stimulates p-IκB and NF-κB via SGK1 in Mesangial Cells**

Next, in experiments designed to determine whether SGK1 activation stimulates the IκB and NF-κB pathways, we found that aldosterone (10 nM) stimulated IκB phosphorylation from 6 h onward in rat mesangial cells (Figure 5, A and B). The aldosterone-induced increments of p-IκB were inhibited by eplerenone (Figure 5, A and B). We also examined the activation of the NF-κB pathway by measuring NF-κB–responsive elements on the basis of luciferase activity. Luciferase activity was significantly increased in rat mesangial cells transfected with plasmids containing NF-κB–responsive elements and reporter gene 12 h after treatment with aldosterone (10 nM). This aldosterone-induced activation of NF-κB was inhibited by transfection with dominant negative SGK1 and siRNA (Figure 5C). We confirmed the expression levels of SGK1 in dom-
Aldosterone Stimulates ICAM-1 Expression via SGK1 and NF-κB in Mesangial Cells

We next investigated the interaction of SGK1 and ICAM-1 expressions. Aldosterone enhanced ICAM-1 promoter activity and the expressions of ICAM-1 protein and mRNA (Figure 6, A through C), and the enhancements in ICAM-1 promoter activity and protein expression were inhibited by transfection with dominant negative SGK1 and siRNA (Figure 6, C, E, and F). To explore whether NF-κB contributes to the aldosterone-stimulated ICAM-1 expression, we coexpressed the ICAM-1

Figure 5. Aldosterone stimulates p-IκB and NF-κB via SGK1 in mesangial cells. (A) Aldosterone (10 nM) was added to the medium with or without eplerenone (10 μmol/L), and the total cellular protein was extracted at the indicate times. p-IκB and actin were analyzed by Western blotting. Similar results were obtained from six replicate experiments. (B) Graph summaries of A using a densitometer. (C) Rat mesangial cells were co-transfected with a luciferase construct containing five NF-κB elements, a control vector (pcDNA3), wild-type SGK1, dominant negative SGK1, or siRNA. The cells were treated with aldosterone for 12 h. (D) Rat mesangial cells were transfected with a control vector (pcDNA3), wild-type SGK1, dominant negative SGK1, or siRNA. The cells were treated with aldosterone for 12 h. p-IκB, t-SGK1, and actin were analyzed by Western blotting. We confirmed the expression levels of SGK1 in dominant negative SGK1- or siRNA-transfected cells. SGK1 expression was significantly induced in wild-type SGK1 and suppressed in siRNA-transfected cells. Aldosterone significantly increased the phosphorylation of IκB, and the increment was inhibited by the transfection with dominant negative SGK1 and siRNA for SGK1. (E) Graph summaries of D using a densitometer. Each bar represents the mean ± SEM (n = 6). *P < 0.05 versus control; # P < 0.05 versus vector.

Figure 6. Aldosterone stimulates ICAM-1 expression via SGK1 and NF-κB in mesangial cells. (A) Rat mesangial cells were treated with aldosterone (10 nM) with or without eplerenone (10 μmol/L) at the indicated times. ICAM-1 and actin were analyzed by Western blotting. Similar results were obtained from six replicate experiments. (B) Graph summaries of the real-time PCR and Western blot analyses of A. (C) Rat mesangial cells were co-transfected with an ICAM-1 promoter construct, a control vector (pcDNA3), wild-type SGK1, dominant negative SGK1, or siRNA. The cells were treated with aldosterone for 12 h. (D) Rat mesangial cells were co-transfected with an ICAM-1 promoter construct, a control vector (pcDNA3), wild-type SGK1, dominant negative SGK1, or siRNA. The cells were treated with aldosterone for 12 h. ICAM-1 and actin were analyzed by Western blotting. Similar results were obtained from six replicate experiments. (F) Graph summaries of E using a densitometer. Each bar represents the mean ± SEM (n = 6). *P < 0.05 versus control; # P < 0.05 versus vector.
promoter with a control vector or a vector encoding a dominant negative mutant of IkBα (IkBDN) known to prevent NF-κB activation. The inhibition of NF-κB activation significantly decreased the ICAM-1 promoter activity (Figure 6D). Eplerenone inhibited aldosterone-induced protein expression and promoter activity of ICAM-1 (Figure 6, A and B).

**Aldosterone Stimulates CTGF Expression via SGK1 and NF-κB in Mesangial Cells**

We also investigated the interaction of SGK1 and CTGF expressions. Aldosterone enhanced CTGF promoter activity and the expressions of CTGF protein and mRNA (Figure 7, A through C). The aldosterone-conferred effects on CTGF protein expression and promoter activity were stimulated by transfection with wild-type SGK1 and inhibited by dominant negative SGK1 and siRNA (Figure 7, C, E, and F). To explore further whether NF-κB contributes to aldosterone-stimulated CTGF expression, we coexpressed the CTGF promoter construct with a control vector or a vector encoding a dominant negative mutant of IkBα (IkBDN) known to prevent NF-κB activation. The inhibition of NF-κB activation significantly decreased the CTGF promoter activity (Figure 7D). Eplerenone inhibited aldosterone-induced protein expression and CTGF promoter activity (Figure 7, A and B).

**Aldosterone Stimulates the Expressions of SGK1, ICAM-1, and CTGF in Glomeruli In Vivo**

Systolic BP (SBP) was significantly higher in aldosterone-treated rats (186 ± 26 mmHg) than in vehicle-treated rats (134 ± 17 mmHg), and that in the former was significantly attenuated by the concurrent administration of eplerenone (148 ± 22 mmHg; n = 10; P < 0.05). Serum aldosterone levels were significantly higher in aldosterone-treated rats (76.6 ± 13.7 ng/dl) than in vehicle-treated rats (11.6 ± 3.1 ng/dl), and that in the former was not significantly changed by the concurrent administration of eplerenone (74.2 ± 17.5 ng/dl; n = 10). The glomeruli in the vehicle-treated rats were normal, whereas those in the aldosterone-treated rats exhibited injury characterized by cellular proliferation and mesangial matrix expansion (Figure 8, A through F). Using Masson trichrome staining, increments of glomerular fibrosis were observed in the aldosterone-treated rats but not in the vehicle-treated rats. The concurrent administration of eplerenone with aldosterone significantly reduced the glomerular fibrosis (Figure 8, G through I). The number of infiltrating macrophages that were positively labeled with the ED-1 antibody was greater in the aldosterone-treated rats than in the vehicle-treated rats. The concurrent administration of eplerenone with aldosterone significantly reduced the number of ED-1–positive cells (Figure 8, J through L). Glomerular mesangial cells that were positive for SGK1 were significantly more frequent in the aldosterone-treated rats (7.8 ± 3.3 cells/glomerular cross-section) than in the vehicle-treated rats (1.5 ± 0.3 cells/glomerulus; P < 0.05; Figure 9, A through C), and the concurrent administration of eplerenone with aldosterone significantly reduced the numbers of SGK1-positive cells in glomeruli (1.8 ± 0.5 cells/glomerulus; P < 0.05; Figure 9M). SGK1 staining was significantly enhanced in cortical collecting ducts by aldosterone (Figure 9, D through F). Glomerular mesangial cells that were positive for ICAM-1 were significantly more frequent in the aldosterone-treated rats (7.5 ± 2.3 cells/glomerulus) than in the vehicle-treated rats (2.1 ± 0.5 cells/glomerulus; P < 0.05; Figure 9, G through I), and the concurrent administration of eplerenone with aldosterone significantly reduced the numbers of ICAM-1–positive cells in glomeruli (3.2 ± 0.6 cells/glomerulus; P < 0.05; Figure 9N). Glomerular mesangial cells
that were positive for CTGF were significantly more frequent in the aldosterone-treated rats (5.7/11006 1.9 cells/glomerulus) than in the vehicle-treated rats (1.7/11006 0.4 cells/glomerulus; P < 0.05; Figure 9, J through L), and the concurrent administration of eplerenone and aldosterone significantly reduced the numbers of CTGF-positive cells in glomeruli (2.3/11006 0.5 cells/glomerulus; Figure 9O). Western blot analyses of isolated glomeruli revealed higher expressions of SGK1, ICAM-1, and CTGF in aldosterone-treated rats than in vehicle-treated rats, and the concurrent administration of eplerenone and aldosterone significantly decreased these expressions (Figure 10).

**DISCUSSION**

In this study, we demonstrated the following effects of aldosterone: Stimulation of SGK1 phosphorylation and SGK1 protein expression in mesangial cells via MR; stimulation of NF-κB mainly via the activation of SGK1; stimulation of ICAM-1 and CTGF transcription, at least in part, via NF-κB and SGK1;
stimulation of SGK1, ICAM-1, and CTGF expressions via MR in vivo. Recent attention has been focused on the role of aldosterone in the pathophysiology of hypertension and cardiovascular disease. The administration of spironolactone markedly ameliorates renal injury in stroke-prone spontaneously hypertensive rats without altering the BP; however, the exact mechanisms responsible for the glomerular inflammation and sclerosis induced by aldosterone remain unclear. Our study is the first to demonstrate that aldosterone stimulates SGK1 expression mainly via the MEK1 pathway and phosphorylates SGK1 via PI3K at Thr-256 in mesangial cells. SGK1 was recently reported to play an important role in the regulation of salt reabsorption by mineralocorticoids in renal distal tubules; however, the pathway by which aldosterone regulates SGK1 in the mesangial cells remains unclear. Our initial experiments in this investigation were designed to examine how the aldosterone-induced expression of SGK1 responded to inhibitors such as PD98051, SB203580, wortmannin, and LY294002 (Figure 3). Of these, PD98051 was the most powerful and significant inhibitor of the aldosterone-induced SGK1 expression in rat mesangial cells. We also demonstrated that the inhibition of the mitogen-activated protein kinase 1/2 cascade by the transfection of dominant negative MEK1 (S222A) significantly abrogated the effect of aldosterone in stimulating SGK1 transcription.

To become functional, SGK1 protein kinase must be activated by phosphorylation. PI3K was recently reported to play a role in the phosphorylation of Thr-256 via insulin and IGF-1. Experiments using LY294002, wortmannin, and the transfection of dominant negative PI3K in this study have demonstrated for the first time that aldosterone phosphorylates SGK1 at Thr-256 in mesangial cells via PI3K. SGK1 kinase activity was significantly activated by aldosterone, and this activation was almost completely inhibited by MR antagonist.

Our results also established that aldosterone activates CTGF transcriptionally in mesangial cells, at least in part, via NF-κB. CTGF is upregulated in numerous fibrotic conditions, including atherosclerosis and fibrosis of the lung, skin, pancreas, liver, and kidney. Evidence suggests that CTGF functions both as a profibrotic marker and as a downstream effector of TGF-β. TGF-β induces CTGF through different signaling pathways and a specific TGF-β-responsive element in the CTGF promoter. According to Han et al., however, spironolactone induces the antifibrotic effects of CTGF through a TGF-β-independent pathway in a mouse model of diabetic nephropathy. According to our results using dominant negative SGK1, siRNA, and dominant negative IκBα, the SGK1 and NF-κB pathways play key roles in aldosterone-induced CTGF upregulation. Vallon et al. recently observed SGK1-dependent CTGF formation in cardiac tissue using SGK1−/− mice. An NF-κB binding site is observed in the CTGF promoter. Recent reports suggested that SGK1 activates NF-κB in breast cancer cells and confers antiapoptotic effects. These data, taken together with our own from this study, suggest that aldosterone stimulates CTGF transcription via SGK1 and, at least partially, via NF-κB in mesangial cells. Our data are also in accordance with previous reports concerning cardiac and renal fibrosis using SGK1−/− mice. Our experiments are also the first to demonstrate that aldosterone stimulates ICAM-1 expression via NF-κB in rat mesangial cells and isolated glomeruli. Studies reported that ICAM-1 expression is upregulated and that ICAM-1 promotes the recruitment of mononuclear cells in rat glomeruli injured by diabetic nephropathy or other glomerular insults. The mechanism by which ICAM-1 expression is regulated in mesangial cells is poorly understood. Kuster et al. found that MR inhibition ameliorates the transition to myocardial failure and attenuates both oxidative stress and ICAM-1 expression in mouse heart. Their report neglected to mention, however, the renal pathology of that model. Our results using dominant negative SGK1 and dominant negative IκBα are the first to demonstrate that the SGK1 and NF-κB pathways play a key role in aldosterone-activated ICAM-1 induction in mesangial cells. The ICAM-1 promoter contains an NF-κB binding site. These data, as well as our own reported here, suggest that aldosterone stimulates ICAM-1 transcription via SGK1 and, at least partially, via NF-κB in mesangial cells.

We also found that MR antagonist treatment ameliorated the glomerular injury and reduced the expressions of SGK1,
ICAM-1, and CTGF in the glomeruli of rats that were treated with aldosterone and 1% NaCl. In similar experiments conducted by Nishiyama et al.,29 MR antagonist ameliorated the glomerular cell proliferation and matrix expansion induced by 1% NaCl and aldosterone. With their model, however, they did not attempt to examine the levels of SGK1, ICAM-1, and CTGF. Although it is often difficult to extend in vitro results to in vivo conditions, our in vivo results are consistent with the aforementioned in vitro studies. Furthermore, Western blot analyses of isolated glomeruli in our study revealed that the aldosterone/1% NaCl treatment significantly enhanced the expressions of SGK1, ICAM-1, and CTGF compared with the levels observed in vehicle/NaCl-treated rats, whereas the concurrent administration of eplerenone significantly reversed this enhancement. The results of these in vivo experiments, however, do not exclude the possibility that the antischlerotic and antifibrotic effects of eplerenone are due, in part, to the mechanical effects of BP on the glomerular and mesangial cells. SBP was significantly higher in the aldosterone-treated rats than in the vehicle-treated rats, and in the former, it was significantly attenuated by a concurrent eplerenone administration. Hypertension itself causes glomerular sclerosis and increases the concentration of proinflammatory cytokines in many animal models. Thus, aldosterone-induced SGK1 expression together with the downstream effects of SGK1 in mesangial cells may not be sufficient to induce glomerular sclerotic and fibrotic changes. This study did not aim to determine the role of MR action on glomerular injury via in vivo studies; further experiments with aldosterone and diuretics are required to elucidate this important issue. However, previous studies showed that aldosterone blockade can provide end-organ protection in severely hypertensive rats in the absence of significant reduction in BP.9,30 Our in vivo and in vitro results suggest that SGK1, ICAM-1, and CTGF may play some role in aldosterone-induced renal fibrosis and inflammation.

This article is the first to report two novel effects of aldosterone in mesangial cells: First, that it stimulates SGK1 expression and protein expression; and, second, that it induces ICAM-1 and CTGF transcription via NF-κB. Among the many signaling pathways potentially involved in aldosterone-induced renal injury (e.g., reactive oxygen species, Rho kinase), we confirmed that the SGK1–NF-κB pathway plays a key role in glomerular fibrosis and inflammation. Further studies will be necessary to extend our understanding of the pathophysiological actions of aldosterone in mesangial cells and glomeruli. Nevertheless, our study suggests that MR antagonists may prove useful as therapeutic targets in the treatment of mesangio proliferative disease.

CONCISE METHODS

Cell Culture
Mesangial cell strains from male Sprague-Dawley rats were isolated and characterized as previously reported.40 The cells (passages 6 through 9) were cultured in an RPMI 1640 medium containing 20% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml selenite at 37°C in a 5% CO2 incubator. In all experiments, the cells were seeded in 10-cm dishes. An HRCE cell line was obtained from Clonetics Cell Systems (Cambrex, East Rutherford, NJ) and maintained in human renal epithelial growth medium as described previously.28 Previous reports confirmed that aldosterone stimulates SGK1 expression in HRCE.28

Animal Preparation
All experimental procedures were performed according to the guidelines for the care and use of animals established by Tokyo Medical and Dental University. Male Sprague-Dawley rats (Clea Japan, Tokyo, Japan) weighing 150 to 200 g were used for the experiments. The rats underwent left uninephrectomy and received 1% NaCl in their drinking solution for 5 wk. Group 1 received 1% NaCl in the drinking solution plus vehicle (0.5% ethanol; n = 10), group 2 received 1% NaCl plus aldosterone (0.75 μg/h, subcutaneously; n = 10), and group 3 received 1% NaCl plus aldosterone plus eplerenone (0.125% in food; n = 10). An osmotic minipump (model 2002; Alza Co., Palo Alto, CA) was implanted subcutaneously in the dorsum of the neck to infuse vehicle or aldosterone. The dosages of aldosterone and eplerenone were determined on the basis of the results of previous studies in rats.28

SBP was measured in conscious rats by tail-cuff plethysmography (BP-98A; Softron Co., Tokyo, Japan). Blood and kidney samples were harvested at the end of week 5. The right kidney (n = 5, each group) was perfused with chilled saline and fixed in 10% buffered paraformaldehyde for histologic examination. The left kidney (n = 5, each group) was used for protein extraction from isolated glomeruli. The renal glomeruli were isolated using a graded sieving technique as described previously. Two hundred glomeruli were used for Western blot analysis, as described previously.41,42 Serum aldosterone levels were determined using a commercial RIA kit (Beckman Coulter, Krefeld, Germany).

Antibodies and Reagents
Anti-rat SGK1, anti–phospho-SGK1 (Thr-256), and anti-mouse MEK1 antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-mouse CTGF, anti-mouse ICAM-1, and anti-mouse actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rat macrophage antibody (ED-1) was provided by Pfizer (New York, NY).

Reverse Transcriptase–PCR
mRNA was extracted from mesangial cells using TRI-REAGENT (Life Technologies, Gaithersburg, MD). PCR was performed using a GeneAmp DNA Amplification Reagent Kit (Perkin-Elmer Cetus, Norwalk, CT). The primers used for CTGF amplification were as follows: Primer 1 (antisense) 5′-GGGAGGGCGAACCCTACTTGTC-3′ and primer 2 5′-CTGTTGCCCCCTGTGACGAT-3′. The predominant cDNA amplification product was predicted to be 139 bp in length. The primers used for ICAM-1 amplification were as follows: Primer 1 (antisense) 5′-TGGGATGGATGGATACCTGA-3′ and primer 2 5′-
TCGAGTGACACAATGGGAA-3’. The predominant cDNA amplification product was predicted to be 135 bp in length. Glyceraldehyde-3-phosphate dehydrogenase was used as a positive control, as previously reported. The PCR products were sequenced to confirm that these bands were actually the predicted cDNA, as described previously.42

**Reporter Constructs, Transient Transfection, and Luciferase Assay**

The ICAM-1 reporter construct used for the luciferase assays contained a human ICAM-1 promoter from residues −1352 to +139 cloned upstream of the luciferase gene (gift from Dr. Paul T. van der Saag).39 The CTGF reporter construct contained a mouse CTGF promoter that had been PCR-amplified from mouse genomic DNA using the primers 5’-CATGCCCAGTCATACCCCTGCTGCC-3’ (forward) and 5’-CATGGGACGCGTGAGGAAGATGCAC-3’ (reverse). The promoter fragment was cloned into pGL3-Basic vector (Promega, Madison, WI). The expression vector pCMV-IκBΔN and a reporter construct containing 5X NF-κB responsive elements cloned upstream of the luciferase gene were obtained from BD Bioscience (San Jose, CA).37 Wild-type MEK1 and dominant negative MEK1 S222A were gifts from Dr. E.G. Krebs.41 Wild-type SGK1 and dominant negative SGK1 (K127M) were gifts from Dr. B. Hemmings.42 Mesangial cells were transfected by electroporation with a previously described method.44

**SGK1 Knockdown Using siRNA**

SGK1 siRNA knockdown was performed by transient transfection with pooled functionally validated SGK1 siRNA (ID#50754; Ambion). Mesangial cells were transfected using the i-Fect siRNA transfection reagent (Neuromics, Northfield, MN). For determination of the transfection efficiency, a Texas Red–labeled siRNA (siGLO RISC-Free siRNA; Dharmacon, Surrey Hills, Australia) was co-transfected with SGK1 siRNA and visualized using fluorescence microscopy. For control siRNA (scrambled sequence) samples, identical conditions were used. For determination of the efficiency of SGK1 knockdown, Western analysis for SGK1 was performed. Several concentrations of SGK1 siRNA (40, 60, and 100 nM) were tested to determine the optimal knockdown conditions.

**Adenoviruses**

Replication-defective, recombinant adenoviruses encoding a wild-type PI3K and dominant negative PI3K were prepared as described previously.44 In brief, the binding site for the catalytic subunit in the plasmid containing a human PI3K regulatory subunit of PI3K was deleted. A control adenovirus vector was also prepared by using a previously described method.44

**Western Blot Analysis, SGK1 Kinase Assay, and Immunoprecipitation**

Whole-cell lysates were extracted from the cultured mesangial cells, and rat-isolated glomeruli were lysed in SDS sample buffer using previously described methods.41,42 Lysate proteins (40 μg/lane) were separated on SDS-polyacrylamide gels and transferred to Immobilon P membranes (Daichikagaku, Tokyo, Japan). The blots were incubated with the indicated antibodies (1:1000 dilution) for 2 h, followed by incubation with horseradish peroxidase–conjugated secondary antibodies (1:2500 dilution) for 1 h. The membranes were visualized using the Amersham ECL system (Amersham, Arlington Heights, IL). For each Western blot, we performed six replicate experiments (Figures 1 through 7). The intensities of the bands were quantified using Molecular Dynamics ImageQuant Software (Sunnyvale, CA). Twenty-four hours after transfection with siRNA and wild-type SGK1, the cells were incubated with aldosterone (10 nM) for 12 h. After incubation, the cells were lysed for 1 h on ice in 150 mM NaCl, 15 mM Tris-HCl, 25 mM NaF, and 1 mM Na3VO4 (pH 8.0). Proteins were immunoprecipitated with anti-SGK1 antibody. An SGK1 activity assay (Stressgen, Ann Arbor, MI) was performed using immunoprecipitated protein according to the manufacturer’s instructions. Twenty-four hours after transfection with siRNA and dominant negative SGK1, the cells were incubated for 2 h in phosphate-free DMEM containing 0.5% BSA and 10 mM HEPES (pH 7.4), followed by further incubation for 2 h in the same medium containing 32Porthophosphate (1 mCi/ml). After incubation, the cells were washed with phosphate-free DMEM and lysed. Proteins were immunoprecipitated with anti-SGK1 antibody. Phosphorylated proteins separated by SDS-PAGE were visualized by autoradiography.

**Statistical Analyses**

All values are expressed as means ± SEM. Statistical comparisons of differences were performed using one- or two-way ANOVA combined with the Newman-Keuls post hoc test. P < 0.05 was considered significant.

**ACKNOWLEDGMENTS**

This work was supported by the Research fund of Mitsukoshi Health and Welfare foundation; Mochida Memorial foundation; Naito Memorial foundation; and a grant from the Ministry of Education, Science, Culture and Sports of Japan (to Y. T).

We thank Dr. EG. Krebs, Dr. Paul T. van der Saag, and Dr. B. Hemmings for providing plasmids.

**DISCLOSURES**

None.

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