Sgk1 Mediates Osmotic Induction of NPR-A Gene in Rat Inner Medullary Collecting Duct Cells

Songcang Chen, James A. McCormick, Kavitha Prabaker, Jian Wang, David Pearce, David G. Gardner

Abstract—We have shown previously that increased extracellular osmolality stimulates expression and promoter activity of the type A natriuretic peptide receptor (NPR-A) gene in rat inner medullary collecting duct (IMCD) cells through a mechanism that involves activation of p38 mitogen-activated protein kinase (MAPK). The serum and glucocorticoid inducible kinase (Sgk) is thought to participate in the regulation of sodium handling in distal tubular segments. We sought to determine whether this kinase might be involved in the osmotic stimulation of NPR-A gene promoter activity. Exposure of cultured IMCD cells to an additional 75 mmol/L NaCl in culture media (final osmolality 475 mosm/kg) resulted in an ∼4-fold increase in Sgk1 protein levels after 7 hours. The Sgk1 induction was almost completely inhibited by the p38 MAPK inhibitor SB203580, indicating that NaCl activates Sgk1 through the p38 MAPK pathway. Transient transfection of a mouse Sgk1 expression vector along with a −1590 NPR-A luciferase reporter resulted in an ∼3-fold increment in reporter activity, which was significantly reduced by cotransfection with a kinase-dead Sgk1 mutant. The NaCl-dependent induction was partially blocked (∼40% inhibition) by cotransfection of the kinase-dead Sgk1 mutant. Neither Sgk1 nor the kinase-dead mutant had any effect on endothelial nitric oxide synthase (eNOS) promoter activity, and the Sgk1 mutant and 8-bromo-cyclic guanosine monophosphate were, to some degree, additive in reducing osmotically stimulated NPR-A promoter activity. Collectively, these data imply that Sgk1 operates over an eNOS-independent, p38 MAPK-dependent pathway in mediating osmotic induction of the NPR-A gene promoter. (Hypertension. 2004;43:866-871.)

Key Words: glucocorticoids ■ kinase ■ receptors ■ atrial natriuretic factor ■ gene regulation ■ signal transduction

The inner medullary collecting duct is the terminal nephron segment responsible for the final regulation of urinary sodium content. It is the target of several hormonal systems that promote either sodium retention or sodium excretion.1 The natriuretic peptides are a family of peptide hormones that promote a natriuretic diuresis in the kidney through a combination of vasoregulatory properties and direct effects on sodium transport in the collecting duct epithelial cell.2 There are 3 types of natriuretic peptides and 3 classes of receptors that signal their activities. Atrial natriuretic peptide and brain natriuretic peptide are produced primarily in the heart (atria and ventricles, respectively), whereas C type natriuretic peptide is produced in a variety of extracardiac tissues including the vascular endothelium. Atrial natriuretic peptide and brain natriuretic peptide are produced primarily in the heart (atria and ventricles, respectively), whereas C type natriuretic peptide is produced in a variety of extracardiac tissues including the vascular endothelium. Atrial natriuretic peptide and brain natriuretic peptide interact primarily with the type A natriuretic peptide receptor (NPR-A), whereas C type natriuretic peptide binds to and activates the type B receptor (NPR-B).2 Each of these receptors is a particulate guanylyl cyclase. Occupancy of the receptor by ligand increases cyclase activity and, thereby, increases cyclic GMP levels in the target cell. In the kidney, it appears to be exclusively NPR-A that signals the natriuretic activity of atrial natriuretic peptide.3

The medullary interstitium that bathes the basolateral surface of the inner medullary collecting duct (IMCD) cell is the only tissue in the body that is almost continuously exposed to a hyperosmotic environment. This increase in tonicity has the potential to control a variety of metabolic processes in these cells, including those responsible for regulating cellular volume.4–6 We have recently shown that increased extracellular osmolality increases the expression of the gene encoding NPR-A in cultured rat inner medullary collecting duct cells. This increase is heavily dependent on an intact p38 mitogen-activated protein kinase (MAPK) signaling system7 and arises, in part (∼50%), from an osmotic suppression of endothelial nitric oxide synthase (eNOS) and consequent reduction in basal cyclic GMP levels in these cells.8 The nature of the NO/cGMP-independent component of the osmo-stimulatory effect has not been defined. As we have noted previously,7,8 the osmo-induction of NPR-A may represent a natriuretic mechanism for preservation of extracellular tonicity in the face of intravascular volume contraction.

The serum- and glucocorticoid-regulated kinase (Sgk) was originally described in mammary epithelial cells9 and subsequently identified as an aldosterone-sensitive gene product in
the tubular epithelial cells of the kidney.\textsuperscript{10} This kinase appears to be a signaling intermediate linking aldosterone and insulin to increased sodium reabsorption in the collecting duct of the kidney. Sgk1 has previously been shown to respond to osmotic stimuli in hepatic epithelial cells as well as a variety of other cell types,\textsuperscript{11–13} leading us to hypothesize that this kinase might serve as an intermediary in the osmotic induction of NPR-A gene expression in IMCD cells. Our studies confirm that Sgk1 is expressed in cultured rat IMCD cells, that this expression is induced by increased extracellular osmolality, and that this, in turn, appears to be linked to the increase in NPR-A gene promoter activity, albeit through an eNOS-independent pathway.

**Methods**

**Materials**

Anti-rabbit Sgk1 antibody was generously provided by Dr Gary Firestone (University of California at Berkeley). RNasy mini kit was obtained from QIAGEN (Santa Clara, Calif). Primer-it RMT kit, hybridization solution, and Nuctrap push columns were purchased from Stratagene (La Jolla, Calif). Other reagents were obtained through standard commercial suppliers.

**Isolation and Culture of IMCD Cells**

Adult Sprague-Dawley female rats were euthanized by CO\textsubscript{2} narcosis followed by bilateral thoracotomy in compliance with a protocol approved by the University of California at San Francisco Committee on Animal Research. The inner medullary tissue from each kidney was dissected free from the outer medulla, minced, and digested with 1 mg/mL collagenase at 37°C with gentle agitation during each 30 minutes cycle. IMCD cells were enriched in the preparation using hypotonic lysis as described previously.\textsuperscript{14} The cells were re-suspended in medium-15 and seeded on to culture plates. After 24 hours, the cells were placed in K-1 medium\textsuperscript{15} without hydrocortisone and cultured for 3 to 4 days.

**Plasmid Constructions**

The wild-type mouse Sgk1 cDNA (nucleotides 17 to 1436, which include the entire open reading frame) was cloned by reverse-transcriptase polymerase chain reaction from 1 \( \mu \)g mouse kidney total RNA using the RNA PCR Core Kit (Applied Biosystems) in accordance with the manufacturer’s instructions, and transferred onto PVDF membranes. The membrane was blocked with 5% nonfat milk in TBST (50 mmol/L Tris-HCl, pH 7.5, 0.1% Tween 20) and probed with a rabbit polyclonal antibody directed against Sgk1. A horseradish peroxidase-conjugated secondary antibody was used to detect immunoreactive bands using the enhanced chemiluminescence (ECL) Western blotting detection system (Amersham Chemical). Signal was identified and quantified using NIH Image.

**RNA Isolation and Northern Blot Analysis**

Primary IMCD cells were treated with 75 mmol/L NaCl in DME H-21/F-12 (1:1) at different time intervals. Total RNA was extracted using the RNaseq minikit, size fractionated by agarose gel electrophoresis, and transferred to membranes. A 1419-bp BamHI/EcoRI fragment of the mouse Sgk1 cDNA was isolated from the Sgk1/pcDNA3 vector, radiolabeled using the primer-it RMT kit, and separated from free nucleotide using Nuctrap push columns. The membranes were hybridized with mouse \(^{32}\)P-labeled Sgk1 cDNA in hybridization solution provided by Stratagene. All membranes were subsequently stripped and rehybridized with a radiolabeled 1150-bp BamHI/EcoRI fragment of 18S rDNA to permit normalization among samples for differences in RNA loading and/or transfer to the filter. Hybridization signals were scanned into a computer and quantified using the National Institutes of Health (NIH) image program.

**Transfection and Luciferase Assay**

Cells were plated in 6-well plates and grown to \(-70\%\) confluence. At that time, transfection was performed with Lipofectin Reagent (Life Technologies) using a protocol recommended by the manufacturer. One \( \mu \)g of \(-1575\) NPR-A-LUC with 0.2 \( \mu \)g CMV-\( \beta \)-galactosidase (\( \beta \)-gal) and different concentrations of Sgk1/pcDNA3 and/or Sgk1/K127M expression vectors were introduced into each well. The DNA-liposome suspension was incubated in the cultures for 5 to 6 hours at 37°C in Opti-MEMI Reduced Serum Medium (Gibco-BRL). The suspension was then removed and replaced with K-1 medium for the ensuing 24 hours, at which point cells were treated with 75 mmol/L NaCl in K-1 medium for 24 hours. Luciferase and \( \beta \)-galactosidase activities were measured. Luciferase levels were normalized for \( \beta \)-galactosidase activity in the individual cultures.

**Western Blot**

The cells were treated with 75 mmol/L NaCl for different time intervals and scraped into lysis buffer.\textsuperscript{9} Ten \( \mu \)g total protein was denatured at 100°C for 3 minutes, subjected to 10% SDS-PAGE, and transferred onto PVDF membranes. The membrane was blocked with 5% nonfat milk in TBST (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.1% Tween 20) and probed with a rabbit polyclonal antibody directed against Sgk1. A horseradish peroxidase-conjugated secondary antibody was used to detect immunoreactive bands using the enhanced chemiluminescence (ECL) Western blotting detection system (Amersham Chemical). Signal was identified and quantified using NIH Image.

**Statistical Analysis**

Data were evaluated using 1-way ANOVA with Newman-Keuls test for significance.

**Results**

Exposure of cultured rat IMCD cells to an additional 75 mmol/L NaCl (final calculated toxicity in the medium=475 mosm/kg H\textsubscript{2}O) resulted in \(-6\)-fold increase in Sgk1 mRNA levels after only 3 hours of treatment (Figure 1A). This increase decreased slightly over the ensuing 5 hours. The induction by NaCl was almost completely inhibited by the p38 MAPK inhibitor SB203580 (Figure 1B), implying that Sgk1 mRNA induction is dependent on a functional p38 MAPK signaling pathway.

The increase in Sgk1 mRNA was accompanied by an increase in Sgk1 protein levels (Figure 2A), which peaked at \(-4\)-fold above basal levels after 7 hours of NaCl stimulation, an appropriate lag behind the accumulation of NPR-A mRNA reflecting the time required for new protein synthesis. The sequential stimulation of Sgk1 mRNA and protein and the delayed time course (7 hours to peak protein accumulation) implies that the bulk of the induction arises from increased synthesis rather than activation of Sgk1. The ability to increase Sgk1 was not confined to NaCl. Addition of
150 mmol/L sucrose, but not 150 mmol/L urea, to the culture medium (final tonicity \(\approx 475\) mosm/kg \(H_2O\)) resulted in a 3-fold increase in Sgk1 protein levels (Figure 2B), indicating that tonicity, per se, contributes to the inductive process. The NaCl-dependent increase in Sgk1 protein levels was almost completely reversed with SB203580 (Figure 2C). SB203580 had a similar effect on sucrose-stimulated Sgk1 protein levels, implying that sucrose and NaCl use a shared osmotic mechanism to drive Sgk1 expression (sucrose: 3.1±0.4 versus sucrose+SB203580: 1.5±0.6; both results expressed as mean fold induction relative to control levels±SD, \(P<0.005, n=3\)).

We have previously shown that osmotic stimulation of IMCD cells results in upregulation of NPR-A promoter activity through a p38 MAPK-dependent mechanism.\(^7\) At least a portion of this stimulation appears to result from osmotic suppression of NO production in these cells.\(^8\) The
reduction in NO levels decreases basal cGMP production and secondarily increases activity of the NPR-A gene promoter. We attempted to determine whether Sgk1 might be involved in mediating at least a component of the osmotic induction of the NPR-A promoter. As shown in Figure 3, cotransfection of an expression vector encoding wild-type Sgk1 together with the −1575 NPR-A luciferase reporter led to 2.5- to 3.0-fold increment in reporter activity at the optimal concentration (1 μg per transfection) of the expression vector. An expression vector encoding a kinase-dead mutant of Sgk1 (Sgk1/K127M) was devoid of activity when cotransfected with −1575 NPR-A luciferase; however, this mutant, when cotransfected along with the wild-type Sgk1 construct, effected a significant reduction in Sgk1-dependent NPR-A promoter activity, implying that the kinase-dead mutant has the ability to function as a competitive inhibitor of wild-type Sgk1 activity. Wild-type Sgk1 had no effect on the NaCl-dependent induction of luciferase activity, implying that Sgk1 is not limiting for the osmotic induction of the NPR-A gene promoter in these cells.

Of note, Sgk1/K127M was capable of partially inhibiting the NaCl-dependent induction of NPR-A promoter activity (Figure 4). Maximal inhibition (~40%) was achieved with 1 to 3 μg of Sgk1/K127M in the transfection. This implies that Sgk1, or a closely related family member, functions as a mediator of the osmo-induction of NPR-A promoter activity in IMCD cells.

This induction appears to be independent of the reduction in eNOS promoter activity that was linked to NPR-A stimulation earlier. Neither wild-type Sgk1 nor Sgk1/K127M at concentrations shown to affect NPR-A promoter activity had any effect on the cotransfected eNOS promoter. Similarly, Sgk1/K127M failed to reverse the NaCl-dependent suppression of eNOS promoter activity (Figure 5A). Furthermore, while Sgk1/K127M and 8-Br-cGMP provided comparable reductions in NPR-A promoter activity, the inhibition was near additive when the two were used together, suggesting that Sgk1/K127M and eNOS/NO/cGMP operate over independent signaling circuitry.

Discussion

Our previous studies have documented that the NPR-A gene is stimulated by increased osmolality in IMCD cells, a finding that may have physiological relevance given the high levels of extracellular toxicity that exist in the medullary interstitium. These studies showed that the osmotic induction of the NPR-A gene promoter was almost entirely dependent on increased p38 MAPK activity and that approximately half of the response resulted from the p38 MAPK-dependent osmotic suppression of eNOS gene expression. The reduction in eNOS was associated with a reduction of basal cGMP levels and subsequent increase in NPR-A promoter activity. The NPR-A gene promoter is inhibited by perturbations that raise cGMP levels in rat aortic smooth muscle cells and IMCD cells.

The present study indicates that: (1) the Sgk1 gene is a target of osmotic induction in IMCD cells; (2) that Sgk1, or a closely related family member, participates in the osmotic induction of the NPR-A gene promoter in these cells; and (3) that Sgk1 does so through a mechanism that does not directly involve suppression of the eNOS gene promoter.

Osmotic induction of the Sgk1 gene product has been reported earlier by Bell et al. By transfecting NmuMg nontransformed mouse mammary epithelial cells with the Sgk1 promoter linked to a chloramphenicol acetyltransferase reporter, they showed that the osmotic stimulation of Sgk1 gene expression was dependent on an increase in Sgk1 gene transcription. They functionally linked this to a Sp1 binding
site in the proximal promoter of the Sgk1 gene. Mutation of this site led to a reduction in promoter stimulation by the osmotic stimulus. This varies somewhat from the more typical osmotic induction of promoter activity involving a specific regulatory element termed the osmotic regulatory element (TGGAAAATTAC18) found in the 5′ flanking sequence of a number of osmotically sensitive genes, including those encoding the sodium/myo-inositol co-transporter,4 sodium/chloride/betaine co-transporter,5 and aldose reductase.6 The use of an Sp1 site to mediate the osmotic induction implies that basal transcription factors can be recruited into a regulatory mode in response to changes in extracellular tonicity. Secondary involvement of osmotic regulatory element, or a related structure, in signaling the osmotic induction of the Sgk1 gene has yet to be demonstrated.

Finally, the fact that Sgk1/K127M has no effect on eNOS promoter activity (implying lack of effect on eNOS expression) and the additivity demonstrated between Sgk1/K127M and 8-bromo-cGMP suggest that Sgk1 traffics largely over the eNOS/cGMP-independent pathway (Figure 6) to control NPR-A promoter activity. Signaling events positioned between p38 MAPK activation and eNOS promoter stimulation remain undefined.

Perspectives
The link between Sgk1 and increased NPR-A gene expression is, on the surface, paradoxical. Activation of Sgk1 in A6 cells by aldosterone10 or insulin19 has been linked to an increase in sodium current in vitro and, inferentially, an increase in sodium reabsorption in the collecting duct of the kidney. Coincident activation of a natriuretic system like the liganded natriuretic peptide receptor seems counterintuitive. However, the role of Sgk1 in sodium conservation remains complex and only partially understood. Targeted knockout of the Sgk1 gene in mice results in no major perturbation in sodium handling.20 This might result from redundancy of function among different members of the Sgk family (Sgk 2 and 3) or it may suggest that Sgk1 plays an important role in controlling signaling pathways involved in retention and excretion of sodium in the distal nephron. Selective activation of one over the other might result from temporal discrepancies in the individual inductive processes (eg, early activation of aldosterone-dependent sodium retention followed by a later stimulation of natriuretic peptide-dependent natriuresis), which would permit greater fine-tuning in the response to a given physiological stimulus. The mechanism underlying the Sgk1-dependent increase in NPR-A promoter activity is unknown; however, given the kinase activity of the former, one would have to postulate the involvement of a phosphorylated intermediate (eg, transcription factor that is activated by a phosphorylation event).

Acknowledgments
Supported by ROIHL45637 (D.G.G.) and RO1-DK56695 (D.P. from the National Institutes of Health).
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Hypertension. 2004;43:866-871; originally published online March 8, 2004;
doi: 10.1161/01.HYP.0000121883.55722.45
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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