Sodium Depletion Enhances Renal Expression of (Pro)Renin Receptor via Cyclic GMP-Protein Kinase G Signaling Pathway

Jiqian Huang, Helmy M. Siragy

Abstract—(Pro)renin receptor (PRR) is expressed in renal vasculature, glomeruli, and tubules. The physiological regulation of this receptor is not well established. We hypothesized that sodium depletion increases PRR expression through cGMP-protein kinase G (PKG) signaling pathway. Renal PRR expressions were evaluated in Sprague-Dawley rats on normal sodium or low-sodium diet (LS) and in cultured rat proximal tubular cells and mouse renal inner medullary collecting duct cells exposed to LS concentration. LS augmented PRR expression in renal glomeruli, proximal tubules, distal tubules, and collecting ducts. LS also increased cGMP production and PKG activity. In cells exposed to normal sodium, cGMP analog increased PKG activity and upregulated PRR expression. In cells exposed to LS, blockade of guanylyl cyclase with 1H-(1,2,4)oxadiazolo(4,3-a)quinazolin-1-one decreased PKG activity and downregulated PRR expression. PKG inhibition decreased phosphatase protein phosphatase 2A activity; suppressed LS-mediated phosphorylation of extracellular signal–regulated kinase, c-Jun N-terminal kinase, c-Jun, and nuclear factor-κB p65; and attenuated LS-mediated PRR upregulation. LS also enhanced DNA binding of cAMP response element binding protein 1 to cAMP response elements, nuclear factor-κB p65 to nuclear factor-κB elements, and c-Jun to activator protein 1 elements in PRR promoter in proximal tubular cells. We conclude that sodium depletion upregulates renal PRR expression via the cGMP-PKG signaling pathway by enhancing binding of cAMP response element binding protein 1, nuclear factor-κB p65, and c-Jun to PRR promoter. (Hypertension. 2012;59:317-323.)

Key Words: (pro)renin receptor ■ sodium depletion ■ cyclic guanidine monophosphate ■ protein kinase G ■ kidney

Prorenin receptor (PRR) is one of the newly discovered components of the renin-angiotensin-aldosterone system and is expressed in renal vasculature, glomeruli, and tubules.1–3 PRR contributes to the conversion of angiotensinogen to angiotensin I.1 Recent studies also demonstrated involvement of PRR in the development of kidney diseases and inflammation.4–7 Overexpression of human PRR in transgenic rats resulted in an increase of aldosterone production and elevation of blood pressure.8 At the present time, the physiological regulation of PRR expression is unknown. The relationship between low sodium intake and increased activity of the renin-angiotensin-aldosterone system is well established. Low sodium intake is associated with increased production of renin9,10 and angiotensin II10–12 and enhanced expression of the angiotensin receptor type 113 and type 2.14 Similarly, a low-sodium diet (LS) enhances renal production of cGMP.15,16–18 Recent studies demonstrated upregulation of PRR in diabetic animals3 and renal cells exposed to high glucose medium.4,5 However, it is unknown whether PRR expression is regulated by sodium or cGMP. Defining the relationship between sodium and PRR could be the first step to elucidate the physiological role of PRR in the kidney. This study was conducted to evaluate whether LS, cGMP, or its messenger protein kinase G (PKG) influences PRR expression. We hypothesized that, in the kidney, sodium depletion enhances PRR expression via cGMP-PKG–mediated intracellular signaling pathway. We also identified the cellular signals, transcription factors, and their functional binding sites in the promoter region of PRR that may influence PRR expression in response to sodium depletion.

Materials and Methods

Animal Preparation, Salt Intake, and Renal Expression of PRR

Study protocols were approved by the University of Virginia Animal Care and Use Committee. Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 245 to 255 g were used in this study. The effects of low-sodium intake on the renal PRR expression were studied by placing the animals for 1 week on normal sodium diet (NS; 0.3% NaCl, Harlan Teklad, Madison, WI) or LS (0.05% NaCl, Harlan Teklad; n=8 for each group). At the end of this period, animals were euthanized, the kidneys were harvested for protein and total RNA extraction, and parts of kidney were also fixed with Bouin fixative. Renal PRR expression was evaluated with...
quantitative real-time PCR, Western blotting, and immunohistochemical staining.

**Cell Culture, Sodium Depletion, and Inhibition of cGMP-PKG Signaling Cascade**

Mouse renal inner medullary collecting duct epithelial cells (IMCDs) were obtained from the American Type Culture Collection (Manassas, VA) and cultured according to American Type Culture Collection recommended protocols. Proximal tubular epithelial cells from Wistar Kyoto rats (proximal tubular cell [PTCs]) were kindly provided by Dr John J. Gildea at the University of Virginia. Cells were grown to confluence in DMEM/Nutrient Mixture F12 (Invitrogen, Carlsbad, CA) supplemented with 10% FCS and antibiotics. Serum starvation was conducted with Opti-MEM I (Invitrogen) for 12 hours.

NS and LS media were prepared according to the methods of Yang et al.19 LS medium was prepared by Opti-MEM I in a 1:1 mixture with 300 mmol/L of d-mannitol (to reduce Na⁺ concentration to 57.03 ± 0.37 mmol/L). In control groups, cells were exposed to Opti-MEM I in a 1:1 mixture with isotonic saline (final Na⁺ concentration was ~132.00 ± 0.32 mmol/L). In time-response course studies, cells were serum-starved for 12 hours previously and then exposed to NS or LS medium for 1, 3, 6, 12, and 24 hours, respectively. At the end of experiments, cells were harvested for total RNA and protein extraction. For different treatments experiments, each drug was added to serum-free medium 30 minutes before the end of serum starvation. After 30 minutes of pretreatment, cells were refreshed with NS or LS medium with or without treatment, which included 1 of the following: cGMP analog (8-bromo-GMP; Calbiochem, La Jolla, CA), 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ; Sigma-Aldrich, St Louis, MO), or PKG inhibitor (Calbiochem).

**Assessing Gene Expression and Protein Phosphorylation**

Determination of gene expression and protein phosphorylation was conducted with real-time PCR and Western blotting assay. The details are as described in the online Data Supplement (please see http://hyper.ahajournals.org).

**Measurement of Cell Viability, cGMP Production, PKG Activity, and Phosphatase Protein Phosphatase 2A Activity**

The measurement of cell viability, cGMP production, PKG, and phosphatase protein phosphatase 2A (PP2A) activities was carried out as described in the online Data Supplement.

**Real-Time Mapping and In Vitro Binding Activities of cAMP Response Element, Nuclear Factor-κB, and Activator Protein 1 Regulatory Elements in the PRR Promotor**

The protocol for real-time mapping of transcription factors cAMP response element binding protein 1 (CREB-1), nuclear factor-κB (NF-κB) p65, and c-Jun to cAMP response element (CRE), NF-κB, and activator protein 1 (AP-1) elements and their in vitro binding activity is detailed in the online Data Supplement.

**Statistical Analysis**

The data analysis was carried out using STATISTICA version 5.0 (StatSoft, Tulsa OK). Results are expressed as mean±SE. Comparisons among different treatment groups were evaluated by ANOVA with repeated measures and the Bonferroni correction method as a post hoc test. A P value of <0.05 was defined as statistically significant.

**Results**

**Sodium Depletion Increased PRR Expression in the Kidneys of Rats**

Compared with NS-treated rats, PRR mRNA and protein expression were upregulated in the kidney of LS-treated rats.

**Sodium Depletion Upregulated PRR Expression in a Time-Dependent Manner in PTCs and IMCDs**

Compared with NS, PRR mRNA and protein were upregulated after 6 hours of LS exposure and reached the peak after 12 hours in PTCs and IMCDs, as demonstrated in Figure S1 (available in the online Data Supplement, at http://hyper.ahajournals.org).

**Sodium Depletion Increased cGMP Production and Relative PKG Activity in PTCs and IMCDs**

cGMP production was significantly increased in culture supernatants and cells of both PTCs and IMCDs after 12 hours.
hours of exposure to LS (Figure S2). cGMP analog, 8-bromo-cGMP, significantly enhanced relative PKG activity in PTCs and IMCDs exposed to NS (Figure S2). LS significantly increased relative PKG activity. Blockade of soluble guanylyl cyclase with ODQ inhibited an LS-induced increase of relative PKG activities in both PTCs and IMCDs (Figure S2).

**Effect of cGMP Stimulation and Soluble Guanylyl Cyclase Blockade on PRR Expression in PTCs**

8-Bromo-cGMP treatment upregulated PRR mRNA and protein expression in PTCs cells exposed to NS (Figure 2A and 2B). There was no dose-dependent effect of 8-bromo-cGMP on PRR mRNA and protein expression at the used doses.

Soluble guanylyl cyclase blockade with ODQ did not influence PRR expression in cells exposed to NS. In contrast, ODQ at the concentration of 100 nmol/L significantly attenuated PRR upregulation in PTCs exposed to LS (Figure 2C and 2D).

**Effect of PKG Inhibition on PRR Expression, PP2A Activities, and Protein Phosphorylation of Signaling Molecules in PTCs**

PKG inhibition did not influence PRR expression in PTCs exposed to NS but attenuated PRR expression in a dose-dependent manner in LS-treated PTCs cells (Figure 3A and 3B). Relative phosphatase PP2A activities were decreased in LS, and this process was reversed by PKG inhibition (Figure 3C).

LS significantly increased the phosphorylation of extracellular signal–regulated kinase (ERK; T185Y187), c-Jun N-terminal kinase 1/2 (T183Y187), c-Jun (S63), CREB-1 (S133), and NF-κB p65 (S276). These protein phosphorylations were attenuated by PKG inhibition (Figure 3C).

**Sodium Depletion Increased CREB-1 Binding to CRE Elements in the PRR Promoter**

Three CREs were predicted in the rat PRR promoter (Figure 4A). Chromatin immunoprecipitation (ChIP) results (Figure 4B through 4D) demonstrated that LS significantly increased CREB-1 binding to all 3 of the CRE elements. Compared with NS, electrophoretic mobility-shift assay results showed increased CREB-1/CRE complex formation and band shift in LS. CREB-1 antibody completely inhibited the formation of CREB-1/CRE complexes (Figure 4E through 4G).

**Sodium Depletion Increased NF-κB p65 Binding to NF-κB Elements in the PRR Promoter**

Two NF-κB binding elements were predicted in the rat PRR promoter (Figure 5A). Results from ChIP and electrophoretic mobility-shift assay demonstrated that LS significantly increased NF-κB p65 binding to the distal NF-κB element (Figure 5B), as well as NF-κB p65/NF-κB complex formation and band shift (Figure 5C). NF-κB p65 antibody completely inhibited the formation of NF-κB p65/NF-κB element complexes (Figure 5C).

**Sodium Depletion Increases c-Jun Binding to AP-1 Elements in the PRR Promoter**

Four AP-1 binding elements were predicted in the rat PRR promoter (Figure 6A). ChIP results demonstrated that LS
significantly increased c-Jun binding to AP-1 elements (Figure 6B through 6E). In contrast to NS, electrophoretic mobility-shift assay results showed increased c-Jun/AP-1 complex formation with LS (Figure 6F through 6I). The c-Jun antibody completely inhibited the formation of c-Jun/AP-1 complexes (Figure 6F through 6I).

Discussion

In the present study, our first finding demonstrated that LS intake significantly upregulated PRR mRNA and protein expression in the rat kidney. Although upregulation of PRR was observed in the whole kidney, it was more pronounced in proximal tubules and collecting ducts. Similarly, in cultured proximal tubular and inner medullary collecting duct epithelial cells, LS exposure also upregulated PRR expression. These results implied that PRR might play a physiological role in proximal tubules and collecting ducts during sodium depletion.

Our in vivo studies were designed to evaluate renal expression of PRR after 1 week of LS diet. However, PRR expression in cultured renal cells was significantly upregulated after 6 hours of LS exposure and reached a peak after 12 hours. These results suggested a rapid change in renal PRR expression in response to changes in sodium depletion. Because of a lack of availability of renal-specific PRR knockout animal model or specific antagonists, we were not able to determine the exact role of this receptor in the regulation of renal functions. Our previous studies demonstrated that PRR is functional because it enhances the intracellular signaling protein phosphorylation and contributes to renal production of inflammatory factors. A possible function of PRR, although not evaluated in the present study, is the regulation of renal sodium handling. A candidate for the link between PRR and sodium reabsorption is ERK. Previous studies demonstrated enhanced ERK phosphorylation by PRR. ERK was shown to regulate the expression/activity of Na+/K+-ATPase, Na+/H+-exchanger, and Na+ channel. Future studies should evaluate the link between PRR and sodium reabsorption.

Despite being exposed to different intratubular sodium concentrations, PRR expression in proximal tubules and inner-medullary collecting ducts is similar in response to low-sodium intake. It is likely that the sensing mechanism of sodium level that influences renal PRR expression may not be directly related to intratubular fluid sodium concentration. Both PTCs and IMCDs increased cGMP production and PKG activity in response to LS exposure. These results suggest that these cells mount similar PRR signaling pathways in response to sodium depletion.

Our second finding was that the increment of cGMP production by sodium depletion contributes to renal PRR expression. Our previous studies showed that LS intake increased cGMP production in the kidneys. In this study, we also demonstrated that LS increased cGMP production in PTCs and IMCDs. Previous studies demonstrated that cGMP is a primary messenger to activate downstream target molecules to initiate secondary response. In this study, PTCs treated with 8-bromo-cGMP in the presence of
NS upregulated PRR mRNA and protein expression. In contrast, inhibition of soluble guanylyl cyclase attenuated PRR expression in cells exposed to LS. These results confirm that PRR is regulated by cGMP.

Similarly, we found that PKG activity was increased in cells exposed to LS and to 8-bromo-cGMP during NS exposure. PKG activity was attenuated by GC inhibition. These results confirm the existence of the LS-cGMP-PKG pathway. The involvement of PKG in the regulation of PRR expression was confirmed by PKG inhibition. PKG inhibition, in a dose-dependent manner, attenuated the observed increase in PRR mRNA and protein expression in cells exposed to LS. These results demonstrated that activated PKG participates in PRR transcriptional regulation in the kidney exposed to LS via the cGMP-PKG signaling pathway.

Multiple CRE, NF-κB, and AP-1 binding elements were found in the PRR promoter. In the present study, we confirmed the involvement of transcription factors CREB-1, NF-κB p65, and c-Jun in LS-stimulated PRR expression. We demonstrated that these regulatory elements were actively functional in PRR promoter in renal cells exposed to LS to enhance this receptor expression. Both ChIP and electrophoretic mobility-shift assay results showed that LS comprehensively enhanced dynamic binding of transcriptional factors.
CREB-1, NF-κB p65, and c-Jun to CRE, NF-κB, and AP-1 elements in vivo and in vitro, respectively. Previously we reported that NF-κB p65 and c-Jun positively enhance transcriptional regulation of PRR expression in renal mesangial cells exposed to high glucose concentration. In this study we further confirmed that CREB-1, NF-κB p65, and c-Jun enhance transcriptional regulation of PRR, and their binding to the PRR promoter was significantly amplified in renal PTCs exposed to LS.

Previous studies demonstrated the involvement of the cGMP-PKG signaling pathway in enhancing the effect of CRE,23,24 NF-κB,25 and AP-126,27 in different tissues or cells. In the present study, kinase phosphorylation of ERK1/2, c-Jun N-terminal kinase, CREB-1, p65, and c-Jun was observed in renal cells exposed to LS. The phosphorylation of these kinases was attenuated by PKG inhibition. These results suggested that PKG mediated the activation of CREB-1, NF-κB p65, and c-Jun and further influenced their binding to regulatory elements of the PRR promoter in renal cells.

Several studies demonstrated that phosphatase PP2A negatively regulates PKC activity in many experimental models.28,29 In this study, LS suppressed phosphatase PP2A activity. This effect was reversed by PKG. Thus, LS seems to decrease phosphatase PP2A and to increase PKC activities. These findings are in agreement with our previous study demonstrating the involvement of PKCs as regulators of mitogen-activated protein kinases and PRR expression in renal mesangial cells.5 Taken together, PKG regulates PRR expression by activating CREB-1, p65, and c-Jun via PKG-PP2A-PKC signaling pathways.

Our previous studies demonstrated that sodium depletion increases renal cGMP concentration by enhancing NO production by angiotensin type II and bradykinin B2 receptors.15,16 Combined with the data in this study, we conclude that sodium depletion upregulates renal PRR expression via the cGMP-PKG signaling pathway and enhancing the activities of transcription factors CREB-1, NF-κB p65, and c-Jun.

**Perspectives**

The present study confirms that sodium depletion significantly upregulates renal expression of the PRR via cGMP-PKG signaling pathways. These findings may help in identifying new mechanisms related to the regulation of this receptor expression under physiological conditions. In addition, this study suggests the possibility of involvement of PRR in the regulation of renal function. Elucidation of this effect could lead to better understanding of the importance of PRR in health and disease.

**Acknowledgments**

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**Disclosures**

None.

**References**


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SODIUM DEPLETION ENHANCES RENAL EXPRESSION OF (PRO)RENIN RECEPTOR VIA cGMP-PKG SIGNALING PATHWAY

Running title: Sodium depletion enhances PRR expression by cGMP-PKG

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Methods

Assessing Gene Expression and Phosphorylation of Proteins

Validation of mRNA changes in the gene expression was achieved by quantitative real-time RT-PCR. Briefly RNA was extracted from kidney tissue and cultured cells with the RNeasy total RNA isolation kit (Qiagen, Valencia CA). The RNA integrity was accessed by 2% formaldehyde agarose gel electrophoresis. Expression level of PRR mRNA was measured by real-time RT–PCR iCycler according to the manufacturer’s instructions (Bio-Rad, Hercules CA). Single-stranded cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad, Hercules CA). PCR was performed with iQ™ SYBR green supermix (Bio-Rad, Hercules CA) according to the manufacturer’s instructions. Primers sequences are listed in Table S1. Reactions were performed in triplicate, and threshold cycle numbers were averaged. None-template control was used as negative control. Samples were calculated with normalization to 18S rRNA.

To analyze the expression and phosphorylation of proteins, whole cell lysates were extracted from kidney tissue or cultured cells with lysis buffer detailed in previous study (3-7). Following primary antibodies against ATP6AP2 (Abcam, Cambridge MA), phospho-Erk 1&2 (pTpY185/187), Erk1/2, phospho-JNK1&2 (pTpY183/185) (Invitrogen, Carlsbad CA), phospho-c-Jun (Ser63), c-Jun, phospho-CREB1(Ser133) and CREB1 (Santa Cruz Biotechnology, Santa Cruz CA), JNK (Cell Signaling Technology, Danvers MA) and β-actin antibody (Sigma, St. Louis MO) were employed. The bands densitometry was performed by ImageMaster™ TotalLab Version 2.0 (Amersham Pharmacia BioTech, Piscataway NJ). The band density of target protein was normalized to the corresponding density of β-actin. The arbitrary unit of band densities was represented as the expression level.

Measurement of Cell Viability, cGMP Production and PKG Activity

Parallel experiments with the same design for measurement of cGMP and PKG activity in PTCs or IMCDs were conducted simultaneously to determine the total cell number in each sample as previously described (4, 7). The total cell number is determined by quantitatively measuring the release of lactate dehydrogenase (LDH) using CytoTox 96 non-radioactive cytotoxicity assay kit (Promega, Madison WI). The total cell number (1 X 10^6 cells) was the average from triplicates and used for the normalization of cGMP concentration, PKG activity and phosphatase PP2A activity of each sample.

The cGMP levels in cell lysate and culture supernants of PTCs and IMCDs were analyzed by cGMP enzyme immunoassay kit (Cayman Chemicals, Ann Arbor MI). Culture supernatant was collected directly for assay. Intracellular cGMP was extracted with 0.1M HCl as per manufacturer’s instruction. Colormetric assay was read at 405nm.

Relative activity of PKG in cellular lysates of PTCs was assessed by colormetric analysis with a CycLex cGMP dependent protein kinase assay kit (MBL International, Woburn MA) according to the manufacturer’s protocol. Briefly, PTCs were harvested and pelleted by centrifugation. Cell pellets were resuspended in extraction buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, 1 X protease inhibitor cocktail). Horseradish peroxidase conjugate of anti-phospho-G-kinase substrate threonine specific antibody, which catalyzes the conversion of the chromogenic substrate tetra-
methylbenzidine from colorless to blue, was employed. The color is quantified by spectrophotometry at 450nm to reflect the relative amount of PKG activity in the sample.

**Real-time mapping of CRE, NF-κB and AP-1 regulatory elements**
Prediction of PRR promoter and transcription factor binding sites were conducted as previously reported (5). We mapped cAMP response element (CRE), activator protein 1 (AP-1) and NF-κB regulatory element (NF-κB) for PRR promoter. Real time mapping of transcription factors CREB-1, NF-κB p65 and c-Jun to CRE, NF-κB and AP-1 elements was conducted with chromatin immunoprecipitation (ChIP) assay kit (Millipore, Billerica MA) as previously described (5). Antibodies against CREB-1, NF-κB p65 and c-Jun (Santa Cruz Biotechnology, Santa Cruz CA) were employed to immuno-precipitate corresponding transcription factors. The appropriated primer pairs corresponding to CRE, NF-κB and AP-1 fragments in the promoter region of PRR gene are listed in Table S1. In each experimental group, triplicate samples were used for statistical analysis.

**In vitro binding activities of CRE, NF-κB and AP-1 to PRR promoter by EMSA**
Electrophoretic migration shift assay (EMSA) was conducted as previously reported (5). Briefly, after 12 hrs of LS medium exposure, nuclear proteins were prepared by NE-PER nuclear and cytoplasmic extraction reagents as per manufacturer’s recommendations. The oligonucleotides probes corresponding to the consensus CRE, NF-κB and AP-1 binding sites in the promoter region of the rat PRR were used to measure the DNA binding activity of CRE, NF-κB and AP-1 and were listed in Table S1. EMSA was performed using a digoxigenin (DIG) gel shift kit (Roche, Indianapolis, IN) as directed by the manufacturer’s instructions. Incubation of antibodies to CREB-1, NF-κB p65 and c-Jun to nuclear lysates prior to labeled probes binding was used for antibody competition experiments with CRE, NF-κB and AP-1, respectively.
<table>
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<th>Target Element</th>
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</tr>
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<td>rat PRR reverse</td>
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<td>GCCCTCACTAAACCACATCCAA</td>
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| Prom-1145F | CCATTCCGAGTGCACTCTCT |
| Prom-1031R | TCTCATCTCTCCCTGCTTTGATTTT |
| Prom-976F | AGGGATGGTTATATGCAGG |
| Prom-808R | ACGGATATCCGAAAATGGA |
| Prom-700F | AATCTTTGGTTCCCTCCTAGC |
| Prom-584R | TTGACCCCATAGGACAAACC |
| Prom-375R | CCACGTTCTAGCCCTTCTG |
| Prom-143R | CCGTACGAGACGGTTATCCT |
| Prom-184F | GGGACGAGAATTTGGGAAC |
| Prom-25R | AGGGAGGGGAATCTGGAG |
| CRE1 | TAGAATGTAGACAGTGACTTTT |
| CRE2 | CAAACAAGTATGCTCTAGTTT |
| CRE3 | TCGTGCAAGATCGCCGCCCAC |
| NF-κB01 | AGTTAAGTAGAAAGTTGCCAGACC |
| NF-κB02 | GATGTCAGAGAGAGCAGTCGACC |
| AP-1.1 | AACTCTCTCTCAGTAAACCTC |
| AP-1.2 | AACAAAGAGATAGTCTGTTAATAACAGG |
| AP-1.3 | GTTCTAGCCCTTCTGACTACG |
| AP-1.4 | ACCGTCTCGTACGGTGAGTAGT |

Table S1 Oligonucleotides employed in RT-PCR, ChIP assay and EMSA
Figure S1. Time-response course studies of PRR expression induced by low sodium in PTCs and IMCD. (A) and (B): PRR mRNA and protein expression in PTCs; (C) and (D): PRR mRNA and protein expression in IMCD. NS: normal sodium; LS: low sodium. Mean is the average of three independent experiments. *p<0.01.
Figure S2. Effects of low sodium and guanylyl cyclase inhibition on cGMP production and relative PKG activity and contribution of cGMP analog stimulation on PKG activity in PTCs and IMCD. NS: normal sodium; LS: low sodium; ODQ: guanylyl cyclase inhibitor (final concentration 100μM). 8-bromo-cGMP refers to cGMP analog 8-Bromo-cGMP. Mean is the average of three independent experiments. *p<0.01.