Online Supplement

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^{TM} 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^{TM} 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 S1 Oligonucleotides employed in RT-PCR, ChIP assay and EMSA

Target Element	Oligonucleotide	Sequence (5' → 3')
	rat PRR forward	TGGCCTATACCAGGAGATCG
	rat PRR reverse	AATAGGTTGCCCACAGCAAG
	mouse PRR forward	TTTGGATGAACT TGGGAAGC
	mouse PRR reverse	CACAAGGGATGTGTCGAATG
	rat 18S rRNA forward	CGAAAGCATTTGCCAAGAAT
	rat 18S rRNA reverse	AGTCGGCATCGTTTATGGTC
	mouse 18S rRNA forward	AATTCCCAGTAAGTGCGGGT
	mouse 18S rRNA reverse	GGCCTCACTAAACCATCCAA
	Prom-1145F	CCATTCCGAGTCACCCTCT
	Prom-1031R	TCTCATCCTCCTGTCTTGATTTT
	Prom-976F	AGGGATGGTATATGCGATGG
	Prom-808R	ACCGAGTATCCGAGAATGGA
	Prom-700F	AATCTTTGGTTCCCTCCTAGC
	Prom-584R	TTGACCCATAGGACAAAAAGC
	Prom-375F	CCACGTTCTAGCCCTTTCTG
	Prom-143R	CCGTACGAGACGGTTATCCT
	Prom-184F	GGGACGAGAATTTTGGAAAC
	Prom-25R	AGGGAGGGGAAATCTGAGAG
	CRE1	TAGAATGTAGACACGTGACTTGTTGTA
	CRE2	CAACAAGTTATGTCATTTAGCTTTTTG
	CRE3	TCGTGCAGATGATCCGCCCGTCCCCAA
	NF-κB01	AGTATAAGTAGAAAGTTCCAGTAGTCGACC
	NF-κB02	GATGCAGAGAGAGGACATCGCCGCATCAAC
	AP-1.1	AACTCCATTCCGAGTCACCCTCTCGGGAAC
	AP-1.2	AACAAAAGATTGGTGTCTAATACACGGGTG
	AP-1.3	GTTCTAGCCCTTTCTGACTACGGGTACAAC
	AP-1.4	ACCGTCTCGTACGGTGAGTAGTGCAATCAG

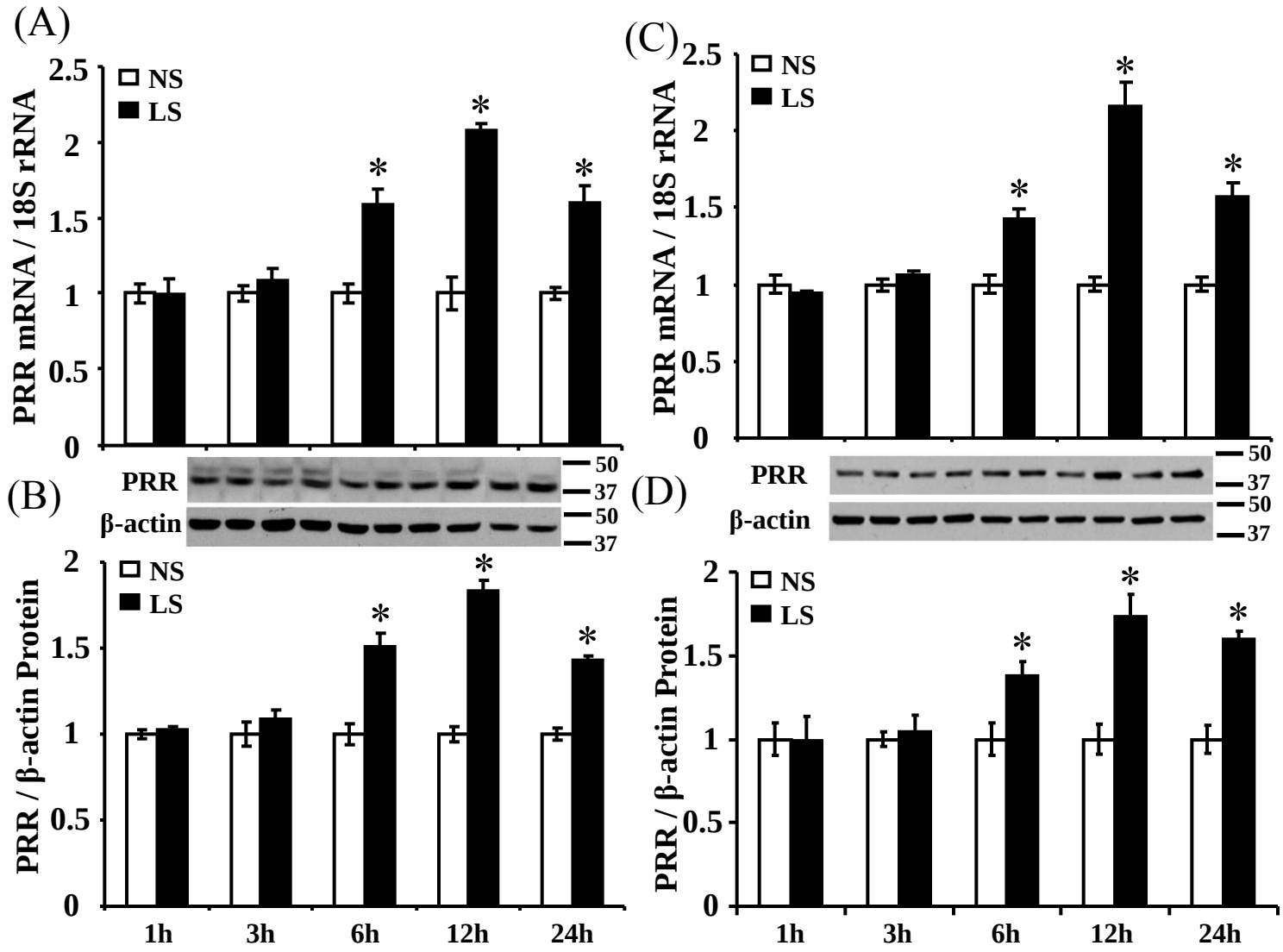

Figure S1. Time-response course studies of PRR expression induced by low sodium in PTCs and IMCD. (A) and (B): PRR mRNA and protein expression in PTCs; (C) and (D): PRR mRNA and protein expression in IMCD. NS: normal sodium; LS: low sodium. Mean is the average of three independent experiments. *p<0.01.

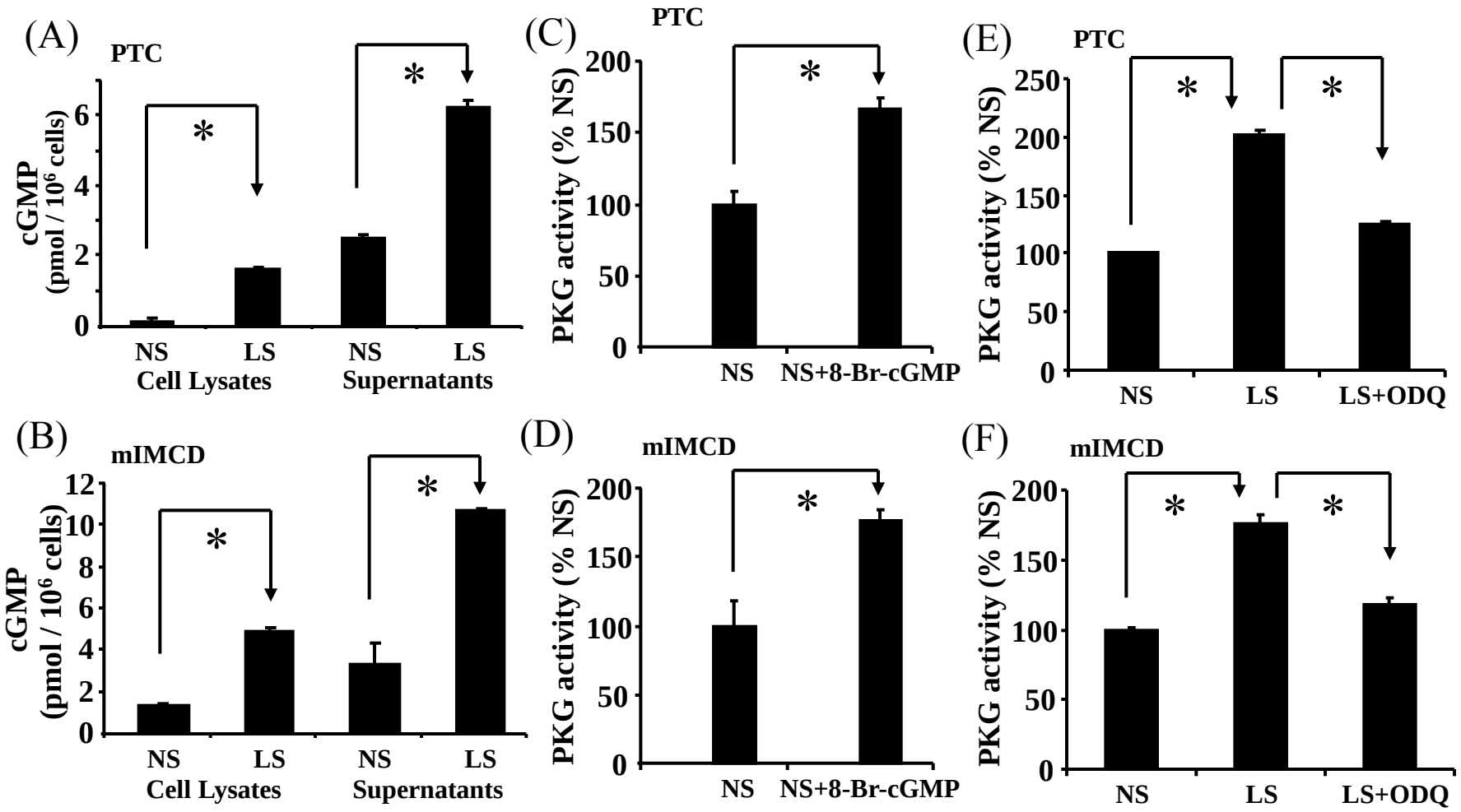

Figure S2. Effects of low sodium and guanylyl cyclase inhibition on cGMP production and relative PKG activity and contribution of cGMP analog stimulation on PKG activity in PTCs and IMCD. NS: normal sodium; LS: low sodium; ODQ: guanylyl cyclase inhibitor (final concentration 100μM). 8-bromo-cGMP refers to cGMP analog 8-Bromo-cGMP. Mean is the average of three independent experiments. *$p<0.01$.