Regulation of (pro)renin receptor expression in mIMCD via the GSK-3β-NFAT5-SIRT-1 signaling pathway

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Quadri S, Siragy HM. Regulation of (pro)renin receptor expression in mIMCD via the GSK-3β-NFAT5-SIRT-1 signaling pathway. Am J Physiol Renal Physiol 307: F593–F600, 2014. First published July 2, 2014; doi:10.1152/ajprenal.00245.2014.—The localization and regulation of (pro)renin receptor (PRR) expression in kidney collecting duct cells are not well established. We hypothesized that low salt (LS) contributes to the regulation of PRR expression in these cells via the GSK-3β-NFAT5-sirtuin1 (SIRT-1) signaling pathway. Mouse inner medullary collecting duct (mIMCD) cells were treated with NaCl at 130 (normal salt; NS), 63 (LS), or 209 mM (high salt; HS) alone or in combination with NaF535. Compared with NS, LS increased the mRNA and protein expression of PRR by 71% and 69% (P < 0.05), and reduced phosphorylation of GSK-3β by 62% (P < 0.01), mRNA and protein expressions of NFAT5 by 65% and 45% (P < 0.05), and SIRT-1 by 44% and 50% (P < 0.01), respectively. LS also enhanced p65 NF-κB by 102% (P < 0.01). Treatment with HS significantly reduced the mRNA and protein expression of PRR by 32% and 23% (P < 0.05), and increased the mRNA and protein expression of NFAT5 by 39% and 45% (P < 0.05) and SIRT-1 by 51% and 56% (P < 0.05), respectively. HS also increased p65 NF-κB by 102% (P < 0.01). Expressions of PRR by 32% and 23% (P < 0.05), and increased the mRNA and protein expression of NFAT5 by 39% and 45% (P < 0.05) and SIRT-1 by 51% and 56% (P < 0.05), respectively. HS also increased p65 NF-κB by 102% (P < 0.01), respectively. LS also increased p65 NF-κB by 102% (P < 0.01). Expressions of PRR by 32% and 23% (P < 0.05), and increased the mRNA and protein expression of NFAT5 by 39% and 45% (P < 0.05) and SIRT-1 by 51% and 56% (P < 0.05), respectively. HS also increased p65 NF-κB by 102% (P < 0.01), respectively. LS also increased p65 NF-κB by 102% (P < 0.01). Expressions of PRR by 32% and 23% (P < 0.05), and increased the mRNA and protein expression of NFAT5 by 39% and 45% (P < 0.05) and SIRT-1 by 51% and 56% (P < 0.05), respectively. HS also increased p65 NF-κB by 102% (P < 0.01), respectively. LS also increased p65 NF-κB by 102% (P < 0.01). Expressions of PRR by 32% and 23% (P < 0.05), and increased the mRNA and protein expression of NFAT5 by 39% and 45% (P < 0.05) and SIRT-1 by 51% and 56% (P < 0.05), respectively. HS also increased p65 NF-κB by 102% (P < 0.01), respectively. LS also increased p65 NF-κB by 102% (P < 0.01). Expressions of PRR by 32% and 23% (P < 0.05), and increased the mRNA and protein expression of NFAT5 by 39% and 45% (P < 0.05) and SIRT-1 by 51% and 56% (P < 0.05), respectively. HS also increased p65 NF-κB by 102% (P < 0.01), respectively.

The (pro)renin receptor (PRR) is a 350-amino acid protein comprising an N-terminal signal peptide, an extracellular domain, a signal transmembrane, and a short cytoplasmic domain. It is localized in various kidney structures, including proximal and distal tubules and collecting ducts (3, 11). Recently, we reported that sodium depletion enhances PRR expression in the kidney (20, 30). The aim of the current study is to expand on this knowledge and evaluate the renal mechanisms that regulate PRR expression induced by low salt (LS).

GSK-3β (GSK3β), a serine/threonine kinase, is ubiquitously expressed and characterized as phosphorylating and inactivating glycogen synthase (22). It is also responsible for regulation of many transcription factors including c-Jun, CREB, β-catenin (23), and NFAT (41). The activity of GSK-3β is inhibited by the phosphorylation of serine residue, particularly at serine 9 (GSK-3β S-9) (23, 48). Previous studies reported that high salt (HS) increases the phosphorylation of GSK-3β S-9, leading to a decrease in the activity of GSK-3β (24) with subsequent upregulation of its target transcription factors. The regulation of GSK-3β under LS conditions is not well elucidated.

Toncinic-responsive enhancer binding protein (TonEBP), also known as nuclear factor of activated T-cells 5 (NFAT5), is a member of the Rel family of transcription factors (14, 17). It contributes to cellular homeostasis in response to hypertonic-induced osmotic stress (14). It has been demonstrated that several mechanisms contribute to the activation of NFAT-5, leading to its translocation from the cytoplasm to the nucleus (7, 26, 32), and transactivation (8), leading to increased mRNA and protein abundance (26, 32). HS increases NFAT5 mRNA and protein expression (5, 48), but very little is known about its regulation under LS conditions.

Sirtuin1 (SIRT-1) is a NAD(+) -regulated deacetylase with numerous known positive effects, including deacetylating substrates involved in metabolism and acute stress resistance (2, 12, 16, 47). SIRT-1 was reported to be present in the renal cortex and medulla and has the ability to regulate blood pressure through inhibition of the angiotensin type 1 receptor (AT1R) (33). SIRT-1 could also directly interact with the p65 component of NF-κB (25, 46) and inhibit NF-κB-dependent gene expression (29, 38, 45). NF-κB has been considered as a proinflammatory transcription factor, and its role in chemokines, the inflammatory cytokines, has been widely studied (27). Recently, we reported that NF-κB p65 is functional in the PRR promoter and enhances PRR expression (20).

The localization of PRR and regulation of its expression by the GSK-3β-NFAT5-SIRT-1 pathway in mouse inner medullary collecting duct (mIMCD) cells represent a novel aspect of how this receptor is regulated in the distal nephron.

MATERIALS AND METHODS

mIMCD epithelial cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and cultured according to ATCC-recommended protocols. Cells were grown to confluence in DMEM/F12 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum and antibiotics. Serum starvation was established for 8 h with Opti-MEM I medium (Invitrogen) before addition of any treatment.

LS media were prepared according to Yang’s method (44). LS medium was prepared by Opti-MEM I medium in a 1:1 mixture with 300 mM d-mannitol (to reduce Na⁺ concentration to 63 mM). In control (normal salt; NS) groups, cells were exposed to Opti-MEM I medium (final Na⁺ concentration 130 mM). HS media was prepared by adding 70 mM NaCl to DMEM/F-12 (Invitrogen) to increase Na⁺ concentration to 209 mM. Osmolality was maintained by adding 300 mM d-mannitol to the culture media and was measured at 290 mosmol/kgH₂O. Cells were cultured in NS, LS, or HS medium for 48 h. At the end of the experiments, cells were harvested for total RNA and protein extraction.

For treatment experiments, cells were starved for 8 h and a potent and selective SIRT-1 inhibitor (EX-527; 100 nM, Santa Cruz Bio-
NFAT5 small interfering RNA transfection. Cells were grown in regular culture medium and transfected with a scrambled NFAT5 small interfering (si) RNA (Qiagen, Valencia, CA) target sequence: 5'-UAUUGUUGACUGAAGUGGtt-3' (sc-38122A: sense: 5'-GUACAGCUAUGCtt-3', antisense: 5'-GCAUAGCUGUUCAGUGAAAtt-3'). Scrambled NFAT5 siRNA was confirmed as nonsilencing double-stranded RNA, and used as a control for mouse NFAT5 siRNA (Santa Cruz Biotechnology). Mouse NFAT5 siRNA is a pool of three different siRNA duplexes with the following target sequences: sc-38122A: sense: 5'-UUGAUGUUGACUGAAGUGGtt-3', antisense: 5'-CCACUUCAGUCAACAUCAAtt-3'; and sc-38122B: sense: 5'-CCAAAGUCAGUCAAAUUCAAtt-3', antisense: 5'-UUUGAAAUUGACUGACUUGGtt-3'; and sc-38122C: sense: 5'-CCACUUCAGUCAACAUAAtt-3', antisense: 5'-UUUGAUGUUGACUGGAGUGtt-3'.

After 6 h of incubation in transfection reagent, the cells were then switched to NS or HS medium as described above.

RNA extraction and RT-PCR. Total RNA extraction was performed using Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA concentration was measured by NanoDrop 1000 (Thermo Fisher Scientific). Single-stranded cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad). PCR was performed with iQTM SYBR green supermix (Bio-Rad) according to the manufacturer's instructions. Primers sequences are as follows: NFAT5: sense 5'-AACATTTGGCACCGCAAAAAGG-3', antisense 5'-GCAACACCCCATCTGTCACTTATA-3'; SIRT-1: sense 5'-CGATGCAGAACGTACACGACG-3', antisense 5'-CTGCAAACCTGTCCAAAGGTA-3'; and PRR: sense 5'-TCTCCGAACTGCAAGTGCTGA-3', antisense 5'-GAGAGCA. Reactions were performed in triplicate, and threshold cycle numbers were averaged. Samples were calculated with normalization to β-actin.

Protein extraction and Western blotting. For protein extraction, cells were washed with cold PBS and subsequently lysed in cold RIPA lysis buffer (Upstate) and a protease inhibitor cocktail (Thermo Scientific). Clear protein extracts were obtained by centrifugation for 20 min at 4°C. Protein concentrations were determined by BCA protein assay, and 20–40 μg of protein mix prepared with loading buffer was loaded per lane. Proteins were transferred to polyvinylidene fluoride (PVDF) membrane filters (Millipore). PVDF membranes were blocked with 5% dry milk for 1 h. Membranes were incubated in the primary antibody overnight at 4°C. The following antibodies were used in Western blotting: PRR (1:1,000 dilution; anti-ATP6IP2/ab40790, Abcam, Cambridge, MA) (9, 30); GSK-3β (1:1,000; Cell Signaling) (39); NFAT5 (1:200 dilution; Santa Cruz Biotechnology) (21); SIRT-1 (1:200 dilution; Santa Cruz Biotechnology) (42); and NF-κB p65 (1:1,000 dilution; Abcam). The membranes were then incubated with the corresponding secondary antibody (1:5,000, horseradish peroxidase-conjugated anti-rabbit) in TBST-5% nonfat milk for 1 h at room temperature, and the immunoreactive bands were visualized by chemiluminescence methods and visualized by X-ray film. Densitometry analysis was performed using Image J software (National Institutes of Health, Bethesda, MD). Protein expressions were normalized to β-actin protein as a loading control.

PRR immunofluorescent and immunohistochemical staining. mIMCD cells were cultured in eight-well chamber slides and treated with NS, LS, or HS medium for 48 h. For immunofluorescence, cells were fixed for 4% PFA for 10 min at room temperature and
incubated with rabbit anti-PRR (1:50 dilution; Santa Cruz Biotechnology) overnight at 4°C. After washing, the slides were then incubated with Alexa Fluor 488-labeled goat anti-rabbit secondary antibody (1:500, Invitrogen) and then mounted with 4,6-diamidino-2-phenylindole-containing mounting solution. The images were captured at ×60 using a confocal laser-scanning microscope (Olympus). For immunohistochemical staining, a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) was used for blocking and color reaction as recommended. The immunostaining images were captured at ×40 using a Qimaging Micropublisher 5.0 RTV camera coupled to a Zeiss Axiophot microscopy (Carl Zeiss, Jena, Germany).

Statistical analysis. Comparisons among different treatment groups were assessed by Student’s t-test (2-tailed) when appropriate or by one-way ANOVA followed by a Tukey test for post hoc comparisons. Data are expressed as means ± SE. P < 0.05 is considered statistically significant.

RESULTS

Expression of PRR in response to NS, LS, and HS. Compared with NS, there was a significant increase in the expression of PRR mRNA and protein by 71% and 69%, respectively (P < 0.05) (Fig. 1, A and B) in cells treated with LS. In contrast, treatment with HS significantly reduced PRR mRNA and protein expression by 32% and 23%, respectively (P < 0.05) (Fig. 1, C and D).

Immunofluorescence for PRR in mIMCD. Compared with NS (Fig. 2, A, D, and G), cells treated with LS demonstrated an increase in the PRR (Fig. 2, B, E, and H). Treatment with HS decreased the expression of the PRR (Fig. 2, C, F, and I) compared with NS and LS.

Fig. 2. Immunostaining of PRR in response to NS, LS, and HS. Negative control IgG (A–C) and immunohistochemistry staining of PRR is shown in brown (D–F; black arrows). Immunofluorescence staining of PRR is shown in green, and 4,6-diamidino-2-phenylindole is shown in blue (G–I; yellow arrows). A, D, and G: NS. B, E, and H: LS. C, F, and I: HS.

Fig. 3. Phosphorylation of GSK-3β in response to NS and LS. Western blot analysis is shown of p-GSK-3β-S-9 treated with NS or LS. Protein levels were normalized to total GSK-3β. Values are means ± SE. *P < 0.05 vs. NS.
Phosphorylation of GSK-3β. Basal phosphorylation of GSK-3β-S9 was detected under NS conditions. Compared with NS, LS treatment significantly reduced the phosphorylation of GSK-3β-S9 by 62% ($P < 0.01$) (Fig. 3).

Expression of NFAT5 in response to NS, LS, and HS. Compared with NS, NFAT5 mRNA and protein expression under LS were significantly reduced by 65% and 45%, respectively ($P < 0.05$) (Fig. 4, A and B). Cells exposed to HS...
demonstrated a significant increase in NFAT5 mRNA and protein expression by 39% and 45%, respectively (P < 0.05) (Fig. 4, C and D) compared with NS.

**Expression of SIRT-1 in response to NS, LS, and HS.** Compared with NS, LS significantly reduced SIRT-1 mRNA and protein expression by 44% and 50%, respectively (P < 0.01) (Fig. 5, A and B). In contrast, treatment with HS significantly increased the SIRT-1 mRNA and protein expressions by 51% and 56%, respectively (P < 0.05) (Fig. 5, C and D).

**NFAT5 expression in NFAT5 siRNA-transfected mIMCD.** Transfection efficiency of NFAT5 siRNA in mIMCD was evaluated by GFP expression using fluorescence microscopy. Figure 6A shows cells in the bright field, and Fig. 6B shows successful transfection of the GFP into the cells.

Posttransfection expression of NFAT5 siRNA was quantitatively measured using RT-PCR. In cells exposed to NS, NFAT5 mRNA or protein expression did not change in response to treatment with scramble or NFAT5 siRNA. Under HS, NFAT5 siRNA significantly reduced the expression of NFAT5 mRNA and protein by 51% and 35%, respectively (P < 0.01) (Fig. 6, C and D) compared with cells treated with HS+ scramble siRNA.

**Expression of PRR in response to NFAT5 siRNA-transfected mIMCD cells.** Under NS, there were no significant changes in the PRR mRNA and protein expression in response to scrambled or NFAT5 siRNA. Treatment with NFAT5 siRNA significantly increased PRR mRNA and protein expression by 148% and 70%, respectively (P < 0.01) (Fig. 6, E and F). Scrambled NFAT siRNA did not influence PRR expression.

**LS and phosphorylation of NF-kB.** Basal phosphorylation of NF-kB was detected under normal conditions. Compared with NS, LS treatment significantly increased the phosphorylation of p65 NF-κB by 102% (P < 0.01) (Fig. 7).

**Expression of PRR in response to EX-527 treatment.** Under NS, treatment with the selective SIRT-1 inhibitor EX-527 did not change PRR mRNA and protein expression. However, during HS treatment, EX-527 significantly increased the expression of PRR mRNA and protein by 96% and 58%, respectively (P < 0.05) (Fig. 8, A and B).

**DISCUSSION**

The present study was designed to investigate the mechanisms contributing to the regulation of PRR in mIMCD by LS.
We hypothesized that salt regulates renal PRR expression by the GSK-3β-NFAT 5-SIRT-1 signaling pathway.

PRR was demonstrated to be expressed in the distal tubule (1), mainly in the medullary collecting duct (37), where it is upregulated by LS (20). LS increases renin secretion and upregulates renin-angiotensin system (RAS) activity in the kidney to regulate sodium and water homeostasis (13, 34). PRR was reported to be colocalized with renin, suggesting an interaction between this receptor and a local RAS in the kidney (11). Indeed, previous studies demonstrated involvement of PRR in renin activation and ANG II generation (35). Our findings of increased PRR expression under LS conditions support the notion that it may play a role in regulation of renal sodium and water homeostasis and blood pressure regulation. Furthermore, increased PRR expression in the kidney may increase epithelial sodium channels via enhancing local RAS activity to stimulate sodium reabsorption in the collecting duct (10). Future studies should evaluate whether PRR plays a role in regulating renal sodium handling.

In the current study, we demonstrated that PRR is expressed in mIMCD cells and its expression is significantly increased by LS. This finding is consistent with our previous finding that in vivo LS intake upregulates PRR expression in the kidney (20). Recently, Wang et al. (40) demonstrated that PRR is expressed in primary rat IMCD and indicated that ANG II upregulates PRR expression. Similarly, we demonstrated regulation of PRR expression by angiotensin subtype AT1R (31). In the present study, experiments were conducted in vitro in mIMCD cells without any addition of exogenous ANG II, suggesting presence of RAS-independent regulation of PRR expression. Collectively, these studies indicate involvement of multiple mechanisms in the regulation of PRR under LS conditions. The increase in PRR expression in response to LS conditions is associated with an increase in its intracellular signals (18,19). Thus it is possible that this receptor could be involved in the regulation of renal function.

In the current study, we demonstrated the reduction in GSK-3β-S9 phosphorylation by LS. Previous studies reported in vivo upregulation of GSK-3β activity during LS via increasing renal ANG II (28). However, our study was conducted in the absence of ANG II. Therefore, the regulation of GSK-3β activity is directly attributed to the salt condition. Enhanced GSK-3β activity was shown to upregulate NFAT5 in HEK293 cells and mIMCD cells (48). Thus the observed reduction in NFAT5 with LS seems to be mediated by decreased GSK-3β-S9 phosphorylation.

Similarly, our findings of LS decreasing the expressions of NFAT5 and SIRT-1 together with previous reports showing increased NFAT5 and SIRT-1 expressions by HS (15, 48) suggest regulation of these signals by salt.

Next, we showed an increase in NF-κB by LS. NF-κB, one of the downstream targets for SIRT-1 (25, 38), is an important transcription factor inducing PRR expression (13). Previous studies demonstrated that SIRT-1 could directly interact and deacetylate the p65 component of NF-κB (25) and inhibit NF-κB activity (43). In our study, we observed that mIMCD exposed to LS showed reduced expression of SIRT-1 and increased phosphorylation of NF-κB p65 (S276).

In summary, LS decreases the phosphorylation of GSK-3β, which downregulates NFAT5, leading to the reduction in SIRT-1 expression. This decrease in SIRT-1 upregulates the
transcription factor p65 NF-κB, a known enhancer of PRR expression. Taken together, the present study demonstrated that salt intake regulates renal expression of PRR via the GSK-3β-NFAT5-SIRT-1 signaling pathway in mIMCD. These findings may lead to a better understanding of new mechanisms contributing to the regulation of PRR expression and renal sodium handling. Future studies are needed to evaluate possible links among PRR, renal disorders, and hypertension.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
Author contributions: S.Q. performed experiments; S.Q. and H.M.S. analyzed data; S.Q. and H.M.S. interpreted results of experiments; S.Q. prepared final version of manuscript.

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