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Soluble (Pro)Renin Receptor as a Negative Regulator of NCC Activity

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Abstract

Sodium-chloride cotransporter (NCC), uniquely located at the distal convoluted tubule (DCT), is the target of thiazide diuretics and critically involved in renal handling of both Na^+ and K^+. However, the mechanism of how NCC activity is regulated remains incompletely understood. Here we report a novel role of (pro)renin receptor (PRR) and its cleavage product soluble PRR (sPRR) via site-1 protease (S1P) as negative regulators of NCC during high salt or high K^+ loading. Under basal condition, mice with DCT-specific deletion of PRR (DCT PRR KO) exhibited modest hypertension associated with reduced urinary Na^+, K^+, and Cl^- excretion due to increased NCC activity. Following a high salt diet, DCT PRR KO mice exhibited a ~25 mm Hg increase of mean arterial pressure contrasting to salt resistance in the floxed controls. The null mice also exhibited impaired kaliuresis and hyperkalemia after high K^+ intake. This phenotype was recapitulated by treatment of C57/BL6 mice with S1P inhibitor PF429242. In cultured Flp-In T-REx 293 NCC cells, S1P-derived sPRR directly dephosphorylated NCC via activation of angiotensin II receptor type 1 (AT1R). Taken together, the present study has demonstrated that S1P-derived sPRR via AT1R negatively regulates NCC activity in the DCT to render salt resistance and to promote K^+ excretion.

Keywords

Soluble (Pro)renin receptor; Sodium-chloride cotransporter; Salt-sensitive hypertension; Potassium; Angiotensin II receptor type 1

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Disclosures

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Introduction

During the past decade, it is well established that the distal convoluted tubule (DCT) plays a central and often dominant role in a variety of homeostatic processes, including extracellular fluid volume regulation, Na^+ reabsorption, and K^+ secretion. The thiazide-sensitive Na^+-Cl^- cotransporter (NCC) is an important player in the maintenance of blood pressure (BP) and Na^+ homeostasis as it controls the fine-tuning Na^+ excretion, which is located in the apical membrane of the epithelial cells of the distal convoluted tubule[1]. NCC is an important pharmacological target in the treatment of hypertension as thiazide-type diuretics, which specifically block NCC, are considered as one of the first-line therapy drugs in the clinic[2, 3], and loss of function mutations in NCC itself causes Gitelman's syndrome[4, 5] and mutations in key regulators of NCC such as With-No-Lysine kinases (WNKs) or Cullin-3 or the BTB (named BTB for *Drosophila* broad-complex C, Tramtrack, and Bric-a-brac) protein kelch-like family member 3 (KLHL3) activate NCC leading to Gordon syndrome[6, 7].

The K^+ secretory process occurs primarily in the aldosterone-sensitive distal nephron (ASDN) where renal outer medullary K^+ channel (ROMK) and calcium-dependent big-conductance K^+ channels (BK) mediate K^+ exit from apical membrane, driven by electrogenic negative charge generated by epithelial sodium channel (ENaC)-mediated Na^+ reabsorption [8]. Increasing evidence indicates that NCC in the DCT plays a key role in regulating K^+ homeostasis and renal K^+ excretion through the control of Na^+ delivery to the collecting duct (CD) [9, 10, 11, 12]. Dietary K^+ intake is a dominant factor regulating NCC activity in animals and humans, low K^+ intake activates NCC, whereas high K^+ (HK) intake suppresses it[13, 14]. More recently, a novel K^+ channel, termed as Kir4.1/Kir5.1, is found on the basolateral membrane of DCT to serve as sensor for extracellular K^+ to participate in K^+ secretion via modulating NCC expression and activity [15].

The renin-angiotensin system (RAS) plays a pivotal role in the maintenance of BP and Na^+ and K^+ homeostasis through complex mechanisms in the ASDN. Indeed, hyperkalemia is a common side effect of RAS inhibition with antagonists against almost each component of the system [16, 17]. (Pro)renin receptor (PRR), a new component of RAS, belongs to the type-I transmembrane receptor family, consisting of a large N-terminal extracellular domain, a single transmembrane domain, and a short cytoplasmic domain [18], and widely expressed in the renal tubules, including proximal tubules, thick ascending limbs, DCTs, and CDs[19, 20]. The extracellular domain is cleaved by a protease to generate 28–kDa soluble PRR (sPRR)[21, 22]. Strong *in vitro* evidence demonstrates that the PRR bound prorenin and renin exhibit increased generation of angiotensin I (Ang I) [23]. *In vivo* evidence also emerges to support PRR function as an important regulator of intrarenal renin activity during HK intake [24] or AngII-induced hypertension[25, 26]. While PRR was initially described as a component of the RAS, its RAS independent role in Na^+ handling via ENaC has also been well demonstrated in both physiological and pathophysiological conditions[26, 27, 28, 29, 30]. However, the role of PRR in NCC regulation in the DCT is completely unknown.

In the present study, we tested the overall hypothesis that PRR/sPPR may function as an integrated regulator of BP and K^+ regulatory pathway through regulation of NCC activity

in the DCT. The experimental approach involves generation of DCT-specific PRR knockout (DCT PRR KO) mice and the use of enzyme inhibitors as well as the use of cultured NCC expressing cell line for the detailed mechanistic investigation.

Materials and Methods

The authors declare that all supporting data are available within the article (and its online-only Data Supplement)

Animals.

DCT-specific PRR KO mice and floxed control mice (3-month old) were all given free access to tap water and fed the standard diet (Na^+: 0.3% and K^+: 1%). Mice were housed in a temperature- and humidity-controlled room with a 12:12-h light-dark cycle. All animal studies were conducted with the approval of the University of Utah Animal Care and Use Committee in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Statistical analysis.

Data are summarized as means ± SEM. Statistical analysis was performed by using one-way analysis of variance (ANOVA) with the Bonferroni test for multiple comparisons, by repeated measures ANOVA for the interaction (time × strain), by unpaired student *t*-test for two comparisons at either different time points or under different conditions/treatments using IBM SPSS 19 software. Difference were considered to be significant when the probability value was less than 0.05.

The rest of the Methods is available in the online-only Data Supplement.

Results

Verification of DCT-specific KO of PRR.

The expression of PV is remarkably restricted to a few cell types in the brain, skeletal, heart muscles, parathyroid glands, and kidney[31]. In the mouse kidney, PV was almost exclusively expressed in the DCT1 so its promoter was successfully used to target DCT1 but not glomeruli with a minimal amount of recombination in thick ascending limbs and other distal tubular segments[31, 32]. PCR of DNA isolated from various organs from DCT PRR KO mouse demonstrated strong recombination in the kidneys with minor recombination in other organs (Figure 1B). It's been shown that PRR mRNA and protein were predominantly expressed in the DCTs and CDs [19]. Therefore, we performed immunofluorescence to detect PRR in the kidney of the two genotypes with co-labeling using anti-AQP2 antibody to visualize principal cells of the CD. The consecutive sections were stained with anti-pNCC-T53 antibody to label DCT. As shown in Figure 1C, PRR was detected in the intercalated cells of AQP2-positive tubules (CD) as well as pNCC-T53-positive tubules (DCT) in floxed mice. In contrast, PRR signal in DCT was markedly reduced in DCT PRR KO mice as compared with floxed controls contrasting to unchanged PRR signal in the CD (Figure 1C), confirming DCT-specific targeting of PRR gene.

DCT PRR KO caused robust salt-sensitive hypertension.

Compared with floxed controls, DCT PRR KO mice exhibited slightly higher BP and greater HCTZ sensitivity under basal conditions (Figure S1). As expected, BPs in floxed mice were largely resistant to HS loading or HCTZ (Figure 2A–C). In a sharp contrast, MAP, SBP, and DBP in DCT PRR KO mice were all robustly induced by HS loading, which were also sensitive to HCTZ (Figure 2A–C). Interestingly, the HR responded similarly to HS loading between the genotypes (Figure 2D). In the setting of HS loading, an interaction (time × strain) was detected for MAP ($p<0.001$, Figure 2A), SBP ($p=0.01$, Figure 2B), and DBP ($p=0.001$, Figure 2C), but not for HR ($p=0.214$, Figure 2D), by using repeated measures ANOVA. In response to HCTZ, the change of MAP, SBP, DBP, and HR was significantly greater in HS-loaded DCT PRR KO mice than that in HS-loaded floxed mice (Figure 2E and 2F).

DCT PRR KO impaired kaliuresis and induced hyperkalemia during HK intake.

As shown in Table S1, DCT PRR KO mice exhibited lower urine volume, accompanied with decreased 24-h urinary Na^+ excretion ($U_{Na}V$), 24-h urinary K^+ excretion (U_KV), and 24-h urinary Cl^- excretion ($U_{Cl}V$) but comparable plasma K^+ concentration (P_KC), plasma Na^+ concentration ($P_{Na}C$), and plasma Cl^- concentration ($P_{Cl}C$). DCT PRR KO mice and floxed controls were treated for 7 days with a NK or HK diet. In response to HK intake, U_KV was increased in both genotypes but the magnitude of the increase was less in DCT PRR KO mice than in floxed controls (Figure 3A). Similar trends were observed for $U_{Na}V$ and $U_{Cl}V$ (Table S1). Following HK intake, DCT PRR KO mice had a greater increase in P_KC (4.40 ± 0.08 mM in KO + HK vs. 3.75 ± 0.05 mM in floxed + HK, $p<0.001$) (Figure 3B). In contrast, $P_{Na}C$ and $P_{Cl}C$ were unaffected by the genotype or the diet (Table S1).

DCT PRR KO promoted NCC phosphorylation.

DCT PRR KO significantly decreased baseline expression of NCC protein (Figure 3C) and mRNA (Figure 3D) but increased baseline abundance of pNCC-T53, and reversed HK-induced down-regulation of pNCC-T53 protein expression (Figure 3C). By immunofluorescence, the expression of pNCC-T53 was localized to the DCT where the signal was enhanced in DCT PRR KO mice as compared with floxed controls under NK condition (Figure 3E). In response to HK intake, the expression of pNCC-T53 in floxed controls was almost undetectable contrasting to the well preserved level in DCT PRR KO mice (Figure 3E). Consistently, HCTZ-induced increases in urinary Na^+ excretion (Figure 3F) were elevated in DCT PRR KO mice as compared with floxed controls under either a NK or HK condition. These results suggest that DCT-specific PRR deletion resulted in increased NCC activity due to enhancement of its phosphorylation despite the reduced expression of total NCC.

In light of the evidence of DCT remodeling accompanied with increased NCC expression[33], we analyzed the density of DCT1 in DCT PRR KO mice in comparison with their floxed controls by performing semi-quantitative immunofluorescence of parvalbumin. We observed that the fractional-area of the DCT1 (Parvalbumin-positive tubules), as reflected by parvalbumin fluorescence intensity, was unchanged in DCT PRR KO mice, revealing no evidence of DCT1 remodeling in these mice (Figure S2). Along this line, gross

kidney morphology as assessed by hematoxylin-eosin staining was normal (Figure S3). Furthermore, we detected the expression of LC3B protein (an autophagosome marker) in the kidney by Western blotting analysis. This result showed no evidence of autophagosome accumulation in DCT PRR KO mice (Figure S4). Overall, the renal structural integrity of DCT PRR KO mice is well preserved.

Examination of WNK4/SPAK/OSR1 pathway.

Abundant evidence supports that the NCC is tightly regulated by the WNK4/Ste20-related, proline–alanine-rich kinase (SPAK)/oxidative stress responsive kinase 1 (OSR1). Thus we detected WNK4, SPAK, and pSPAK/pOSR1 protein expression by immunoblotting analysis. The expression of WNK4 and pSPAK-S373/pOSR1-S325 was higher in DCT PRR KO mice than that in floxed controls (Figure S5). In response to HK intake, renal expression of WNK4 and pSPAK-S373/pOSR1-S325 expression was upregulated in floxed mice and this upregulation was not obvious in DCT PRR KO mice likely due to their elevated baseline values (Figure S5)

DCT PRR KO blocked the cleavage of γ-ENaC during HK intake.

ENaC, a major Na^+ transporter in the epithelial cells of the CD, drives K^+ secretion by mediating Na^+ reabsorption. We examined renal expression of the 3 subunits of ENaC, α, β, and γ, in the floxed and DCT PRR KO mice on a HK diet by immunoblotting analysis. During the normal diet, DCT PRR KO slightly increased β-ENaC protein expression with no effect on α-ENaC or γ-ENaC protein expression in the renal cortex. In response to HK diet, protein expression of ENaC subunits exhibited distinct responses with increases in β-ENaC expression and cleavage of γ-ENaC but not α-ENaC protein expression. DCT PRR KO significantly blocked HK-induced cleavage of γ-ENaC (Figure S4).

PF429242 impaired kaliuresis, increased plasma K^+ concentration, and reversed HK-induced inhibition of NCC.

Recent studies from two independent laboratories consistently demonstrated that S1P served as a predominant enzymatic source of sPRR [21, 22]. Here we examined the potential role of S1P-derived sPRR in regulation of K^+ homeostasis during HK intake. C57BL/6 mice were treated for 3 days with a NK diet, a HK diet alone or in combination with a S1P inhibitor PF429242. As compared with the NK control, HK mice exhibited increased 24-h U_KV (Figure 4A) and slightly elevated plasma K^+ concentration (Figure 4B). In contrast, HK + PF mice had a blunted kaliuretic response and a greater increase in P_KC (HK + PF: 4.56±0.16 mM *vs.* HK: 4.10 ± 0.11 mM, $P<0.01$) (Figure 4B). PF429242 treatment significantly inhibited HK-induced down-regulation of protein expression of NCC and pNCC-T53 (Figure 4C), as well as NCC mRNA (Figure 4D). Immunofluorescence for pNCC-T53 also showed that PF429242 enhanced the expression of pNCC-T53 in the DCT as compared with the HK group (Figure 4E). The *in vivo* NCC activity as reflected by HCTZ-induced increases in urinary Na^+ excretion (Figure 4F) was significantly attenuated by HK intake, which was nearly completely reversed by PF429242.

sPRR dephosphorylated NCC via the activation of AT1R in the Flp-In T-Rex 293 NCC cell.

The Flp-In T-Rex 293 NCC cell line expresses NCC and pNCC-T53 protein following tetracycline treatment and has been used to investigate the mechanism of NCC regulation[34]. To test a direct effect of PRR on NCC expression, we performed an *in vitro* experiment using stably transfected HEK293 cells, Flp-In T-REx 293 NCC cell line, in which the NCC expression was induced by tetracycline. The abundance of NCC and pNCC-T53 protein was significantly increased after tetracycline incubation (Data not shown). As shown in Figure 5A, PRR knockdown by siRNA increased, whereas prorenin treatment decreased, pNCC-T53 abundance in these cells, without affecting total NCC abundance, suggesting a phosphorylation event affected by sPRR.

The cells exposed to PF429242 exhibited reduced abundance of endogenous sPRR as the 28 kDa band accompanied with increased abundance of fPRR, evidence of inhibition of PRR cleavage (Figure 5B). The size of sPRR-His, predicted to be of 29.6 kDa, was detected as a band of a slightly higher molecular weight band as compared with the endogenous sPRR (Figure 5B). sPRR-His treatment decreased, whereas S1P inhibitor PF429242 increased pNCC-T53 abundance without affecting total NCC abundance (Figure 5B). These results suggest a potential role of sPRR in regulation of phosphorylation but not expression of NCC. Given the observation in another study that sPRR directly interacts and activates AT1R in endothelial cells[35], we wondered whether sPRR may act in RAS-dependent manner and therefore examined the effect of inhibition of AT1R as well as mineralocorticoid receptor on the action of sPRR-His. AT1R inhibition by losartan or siRNA (Figure 5C&D) but not mineralocorticoid receptor antagonist eplerenone (Figure 5E) effectively reversed sPRR-His-induced down-regulation of pNCC-T53. To provide the direct evidence of sPRR regulation of NCC activity, we performed functional studies using a membrane-permeable fluorescent intracellular sodium indicator CoroNa™ Green (CoroNa) to evaluate NCC-mediated sodium retention in Flp-In T-REx 293 NCC cells, in which the portion of thiazide-inhibitable fluorescence will reflect NCC activity as reported previously[36]. As shown in figure 5F, the NCC activity was induced nearly 18-fold after the induction of NCC expression by tetracycline, which was significantly blunted by sPRR-His.

Given the inconclusive data on WNK/SPAK/OSR1 pathway, we turned our attention to phosphatase, a potential target of sPRR-mediated dephosphorylation of NCC. Therefore we detected the phosphatase activity[37] in Flp-In T-REx 293 NCC cells exposed to sPRR-His treatment. The phosphatase activity (Figure S6) in the cell lysates was increased following sPRR-His treatment, which was blocked by losartan or AT1R siRNA.

Discussion

The importance of NCC in renal control of Na^+ and K^+ homeostasis has been well established. Relatively, less is known about the molecular mechanism of balanced control of the activity of NCC. While there is overwhelming information concerning phosphorylation and activation of NCC by WNKs, the present study is the first to show that S1P-derived sPRR plays an important role in negative regulation of phosphorylation of NCC and thus salt sensitivity during high salt intake and kaliuretic response to HK loading. DCT PRR KO mice exhibited enhanced salt sensitivity during high salt intake and impaired kaliuresis

during HK intake, accompanied with enhanced pNCC-T53 abundance. This phenotype was recapitulated by treatment of C57/BL6 mice with S1P inhibitor PF429242. In cultured Flp-In T-REx 293 NCC cells, S1P-derived sPRR directly dephosphorylated NCC in RAS-dependent manner.

A large body of experimental evidence has demonstrated an essential role of PRR in renal control of fluid and electrolyte balance and BP. In general, overactivation of PRR promotes Na^+ and water retention and the development of hypertension. The renal action of PRR primarily involves enhancement of expression of various transporters and their associated proteins such as AQP2, vasopressin receptor type 2, α-ENaC, Na/K/2Cl cotransporter, and Na/H exchanger 3 implicated in regulation of urine concentrating capability[38, 39, 40, 41] or the development of hypertension induced by AngII infusion[26, 30] or fructose/salt treatment[42, 43]. The present study is the first to show NCC as an additional molecular target of PRR. Contrary to the overall stimulatory effect of PRR on renal tubular transport mediated by multiple Na^+ and water transporters, we observed an inhibitory effect of PRR/sPRR on NCC activity. In this regard, *in vivo* NCC activity as assessed by the diuretic response to thiazide diuretics was elevated in DCT PRR KO mice, accompanied with Na^+ and water retention and modest hypertension under basal condition. Following HS loading, the null mice exhibited a robust increase in BP, which was sensitive to thiazide diuretics. In parallel with the enhanced NCC activity, renal pNCC-T53 level was elevated in the null mice. These mice were not only salt sensitive but also exhibited impairment of kaliuretic response leading to hyperkalemia during HK intake, thus mimicking familial hyperkalemic hypertension, also known as Gordon's syndrome, a genetic disorder caused by overactivation of NCC due to mutations in genes encoding WNK1 and WNK4 or components of an ubiquitin ligase complex, cullin3, and KLHL3[44]. Of note, salt-sensitive hypertension in DCT PRR KO was only partially blunted by HCTZ, indicating that dysregulation of NCC is not solely responsible for the hypertensive phenotype. Indeed, we observed a small but significant increase in β-ENaC protein expression in the null mice. Future studies are needed to examine if other Na^+ transporters beyond distal nephron are also affected.

We further provide evidence for S1P-derived sPRR in mediating the inhibitory effect of PRR on phosphorylation of NCC. In particular, *in vitro* data demonstrated a direct inhibitory effect of sPRR on phosphorylation of NCC without an effect of total NCC abundance. Consistently, sPRR significantly inhibited tetracycline-induced HCTZ-sensitive sodium uptake in Flp-In T-REx 293 NCC cells, representing direct evidence of sPRR-dependent regulation of NCC activity. Overall, these results strongly suggest that PRR via its S1P-mediated generation of sPRR tonically dephosphorylates NCC to inhibit its activity to render salt resistance and to promote K^+ excretion. This pathway has never been reported by prior studies. It is interesting to note that despite increased NCC activity, NCC mRNA and protein expression was downregulated in DCT PRR KO mice. The mechanism for the later phenomenon is unknown. The downregulation of NCC expression might be due to a compensatory response to the altered NCC activity. In support of this possibility, *in vitro* data showed that sPRR only regulated phosphorylation but not expression of NCC. In support of this notion, there are a number of examples of altered phosphorylation of NCC

occurs contrasting to unchanged total NCC during acute K^+ loading[45], circadian regulation of BP and renal function[46, 47].

The phosphorylation status of NCC is determined by the balanced action of protein kinases, namely the WNK/SPAK/OSR1 pathway, and phosphatases. The activated WNK/SPAK/OSR1 pathway presumably accounts for increased p-NCC level in the kidney of DCT PRR KO mice under basal condition. However, to our surprise, HK intake stimulates but not inhibits the WNK/SPAK/OSR1 pathway in either floxed controls or DCT PRR KO mice, a pattern totally inconsistent with that of p-NCC. This result is consistent with the previous reports by Hoorn group[48]. Thus, these results may suggest that the WNK/SPAK/OSR1 pathway may not be a primary determinant of p-NCC abundance at least during HK intake. This may leave room for participation of phosphatases. Indeed, we found sPRR-His stimulated phosphatase activity in Flp-In T-REx 293 NCC cells in a AT1R-dependent manner. These results suggest that sPRR may directly enhance phosphatase activity to dephosphorylate NCC via AT1R signaling. The identity of such phosphatase awaits future investigation.

In vitro experiments using a Flp-In T-REx 293 NCC cell line demonstrated dependence of sPRR action on AT1R. In support of this notion, inhibition of AT1R with losartan or siRNA consistently blocked of the inhibitory effect of sPRR-His on pNCC-T53 abundance. These results are compatible with increasing evidence supporting intrinsic association between PRR/sPRR and the RAS. In particular, PRR/sPRR have been shown to be critical regulators of intrarenal RAS during pathogenesis of hypertension[49] and chronic kidney disease[50]. In primary cultured DCT cells, AngII–increased Na-22 transport was inhibited by AT1R antagonist losartan[51]. In line with these functional studies, immunoreactivity of AT1aR was detected throughout the kidney, including the DCT and CD[52, 53, 54]. Using a micro-assay that permits measurement of hormone binding in discrete tubule segments, the binding sites of ^{125}I-AngII were presented in all tubule segments including DCT and CD[55]. These studies demonstrated the presence of AT1R in the DCT that regulates NCC. Our observation also agrees well with upregulation of expression and activity of NCC in male AT1Ra KO mice [56], although AngII infusion is shown to stimulate NCC expression, phosphorylation [57], and trafficking [58]. The discrepancy may result from confounding influence of non-physiological doses of AngII or the distinct actions of AT1R versus AT2R.

Aldosterone is well known to be involved in renal handling of both Na^+ and K^+ through its action in the distal nephron. Aldosterone paradox highlights its distinct physiological roles under hypervolemia and hyperkalemia conditions. During volume depletion, aldosterone primarily regulates Na^+ reabsorption in the distal nephron with minimal influence on K^+ secretion whereas during hyperkalemia aldosterone exerts a predominant role in promoting K^+ secretion. In the present study, we discovered kaliuretic action of S1P-derived sPRR possibly via activation of AT1R in the presence of suppressed AngII level. In contrast, during volume depletion, circulating AngII is elevated to activate AT1R to drive overactivation of the RAAS. However, renal expression of PRR is paradoxically upregulated by both salt depletion and high-salt loading [59, 60] and production of sPRR under either condition is unclear. Based on the available knowledge, we would like to postulate that AT1R may be selectively activated by sPRR during hyperkalemia to promote kaliuresis and

by AngII during volume depletion to retain Na^+, thus at least, in part, explaining aldosterone paradox.

The present study has a number of limitations. For example, the specificity of PV-Cre-mediated gene recombination is limited. Despite the strict restriction of high PV abundance to the DCT1 in the mouse kidneys [32, 61], the expression is also detected in extrarenal tissues such as the neurons. Indeed, we observed modest recombination event in the brain, which may have some confounding influence on the renal phenotype. It is been shown that neuron-specific PRR KO or neuron PRR inhibition prevents the development of salt-sensitive hypertension or AngII-induced hypertension[62, 63, 64, 65, 66], suggesting pro-hypertensive action of central PRR, which unlikely accounts for the antihypertensive action of DCT PRR as shown by the present study. Another limitation is that the activity of PV-Cre is mostly restricted to DCT1 but not DCT2. Therefore, a potential role of PRR in DCT2 remains uninvestigated by the current study. This issue can soon be resolved by the use of a newly generated NCC-Cre that targets the entire DCT [67].

In summary, the present study comprehensively examined the role and mechanism of DCT PRR in regulation of NCC activity during renal control of salt sensitivity and K^+ homeostasis. PRR via its S1P-mediated cleavage product sPRR tonically dephosphorylate NCC to suppress its activity to coordinate Na^+ retention and K^+ excretion. Overall, these results help define S1P-derived sPRR as a negative regulator of NCC in the DCT.

Perspectives

Accumulating evidence our group and others has defined PRR as important regulator of renal function, particularly Na^+ and water transport in the collecting duct[26, 30, 39]. However, there is no prior report on the potential function of PRR in the DCT where NCC-mediated Na^+ transport plays a key role in regulation of both Na^+ and K^+ transport. The current work showed that S1P-derived sPRR suppresses NCC activity in the DCT to render salt resistance and to promote K^+ excretion. The results suggest an essential and non-redundant role for sPRR in modulating NCC activity. This finding provides a rationale for the integrated mechanism of renal control of salt sensitivity and K^+ homeostasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Novelty and Significance

What Is New?

- We for the first time demonstrate that PRR via its S1P-derived soluble sPRR functions as a key negative regulator of NCC activity for controlling blood pressure and K^+ homeostasis.

What Is Relevant?

- DCT PRR KO mice exhibit salt-sensitive hypertension and impaired K^+ excretion due to enhanced NCC activity, mimicking a human genetic disease of Gordon's syndrome.
- Thiazide diuretics are widely used in the clinical practice for management of hypertension and other fluid retention status via inhibiting NCC. It is of high clinical significance to understand the signaling pathways that regulate this Na^+ transporter.

Summary

- We have successfully generated a novel mouse model with conditional deletion of PRR in the DCT and observed a clinically relevant phenotype during high salt and high K^+ intake. We have further provided evidence for involvement of S1P-derived sPRR in negative regulation of NCC activity. Therefore, we have identified a previously undescribed PRR/sPRR-dependent pathway in the DCT during renal handling of Na^+ and K^+.

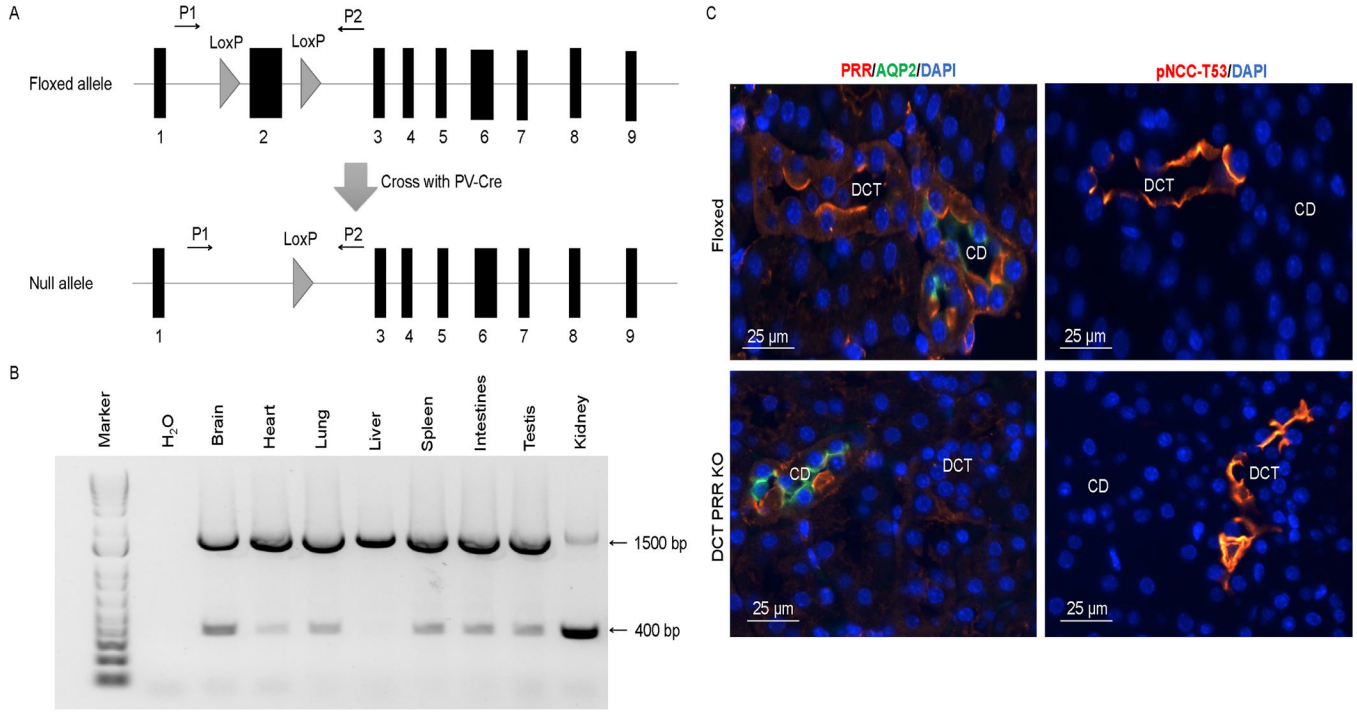

Figure 1.

Gene-targeting strategy and parvalbumin-Cre-mediated recombination in DCT-specific PRR knockout (DCT PRR KO) mice. (A) Construct wherein exon 2 of the PRR gene, flanked by loxP sites, is removed in the presence of Cre recombinase. (B) Representative blot of PRR gene recombination in various organs in DCT PRR KO mice. The top 1,500-bp band represents the unrecombined allele, and the bottom 400-bp band the recombined allele. (C) Immunofluorescent staining of PRR and pNCC-T53 in the kidneys of floxed control or DCT PRR KO mice.

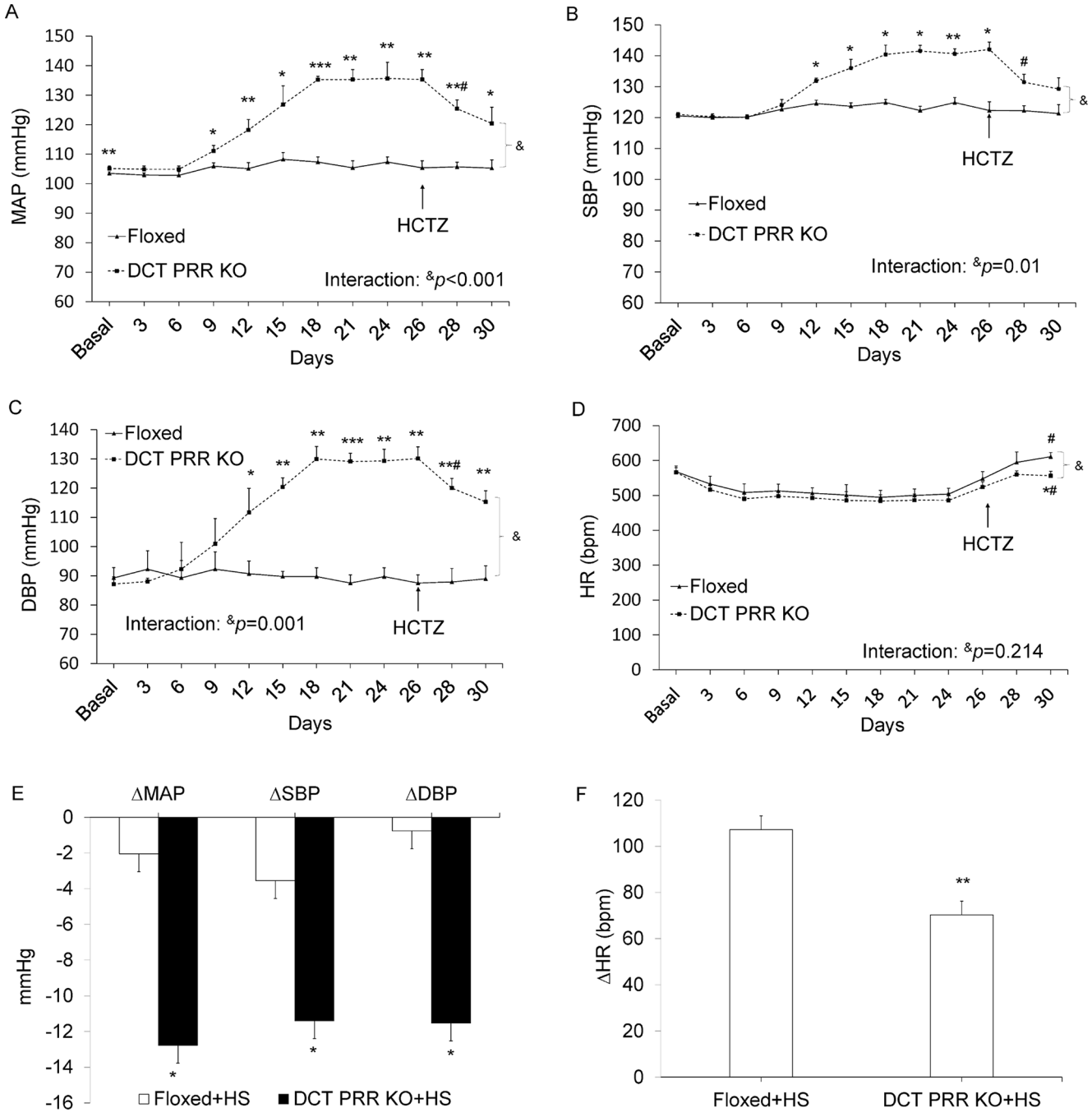

Figure 2.

BP analysis of DCT PRR KO mice during high Na^+ (HS, 4% NaCl) diet treatment. (A-F) BP was monitored daily by radiotelemetry following a HS diet. At day24, both floxed and DCT PRR KO mice were all subjected to hydrochlorothiazide (HCTZ) (100 mg/L added to drinking water) treatment for 6 days. (A) MAP, (B) SBP, (C) DBP, (D) HR. Data are means ± SEM; n = 4 per group. $^{\&}p<0.001$ (C), $^{\&}p=0.01$ (D), $^{\&}p=0.001$ (E), and $^{\&}p=0.214$ (F) (analysis of the interaction [time × strain] by repeated-measures ANOVA). $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ *vs.* Floxed at the corresponding time period (unpaired Student's *t*

test); $^{\#}p<0.05$ *vs.* Day 24 (unpaired Student's *t* test). (E, F) Change in ΔMAP, ΔSBP, ΔDBP (E), and heart rate (ΔHR) (F) after HCTZ treatment for 6 days, determined by the delta value of BP or HR at Day24 and Day30. Data are means ± SEM; n = 4 per group. $^{*}p<0.05$, $^{**}p<0.01$ *vs.* Floxed + HS (unpaired Student's *t* test).

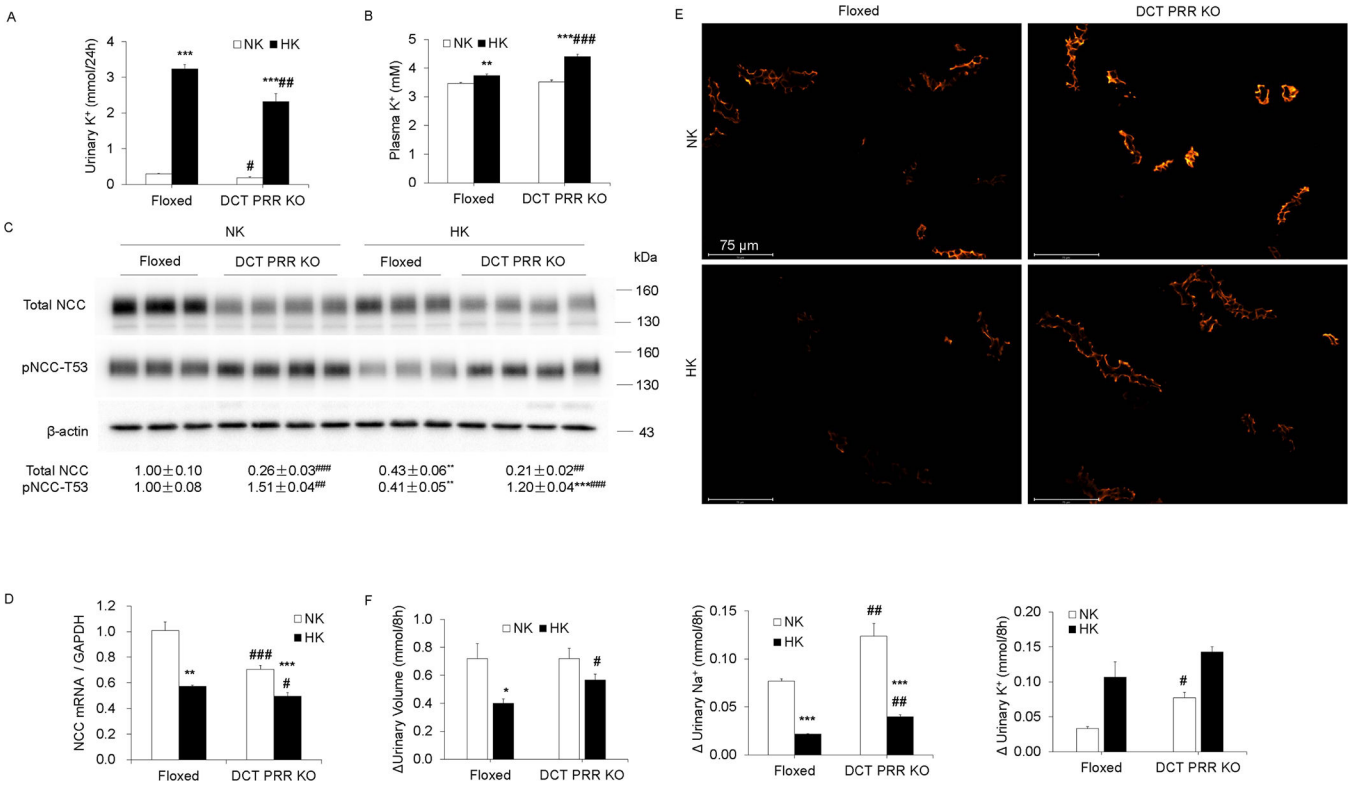

Figure 3.

Analysis of plasma K^+ concentration, urinary K^+ excretion, and pNCC in NK and HK-loaded floxed and DCT PRR KO mice. (A, B) Urinary K^+ excretion (A) and plasma K^+ concentration (B) in NK and HK- loaded floxed and DCT PRR KO mice. $^{**}p<0.01$, $^{***}p<0.001$ *vs.* NK; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ *vs.* Floxed (ANOVA with the Bonferroni test). (C) Immunoblotting and densitometric analysis of NCC and pNCC-T53 in the renal cortex in NK and HK-loaded floxed and DCT PRR KO mice. A separated membrane was probed with β-actin as an internal control for equal loading of the samples. N = 5 per group. Data are mean ± SEM. $^{**}p<0.01$, $^{***}p<0.001$ *vs.* NK; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ *vs.* Floxed (ANOVA with the Bonferroni test). (D) Representative immunofluorescence images of pNCC-T53 in DCT of NK and HK-loaded floxed and DCT PRR KO mice. The images shown are representatives of 5 animals per group. (E) *In vivo* NCC activity as reflected by rapid diuresis and natriuretic responses to hydrochlorothiazide (HCTZ). NK and HK-loaded floxed and DCT PRR KO mice were all subjected to a single dose of vehicle or HCTZ (10 mg/kg by gavage) treatment, followed by 8-h urine collection, and shown was the change in 8-h urinary Na^+ and K^+ excretion, determined by the delta value of 8-h urinary Na^+ or K^+ excretion of vehicle and HCTZ treatment. N = 5–15 per group. Data are mean ± SEM. $^{*}p<0.05$ and $^{***}p<0.001$ vs. NK; $^{\#}p<0.05$ and $^{\#\#}p<0.01$ vs. Floxed (ANOVA with the Bonferroni test).

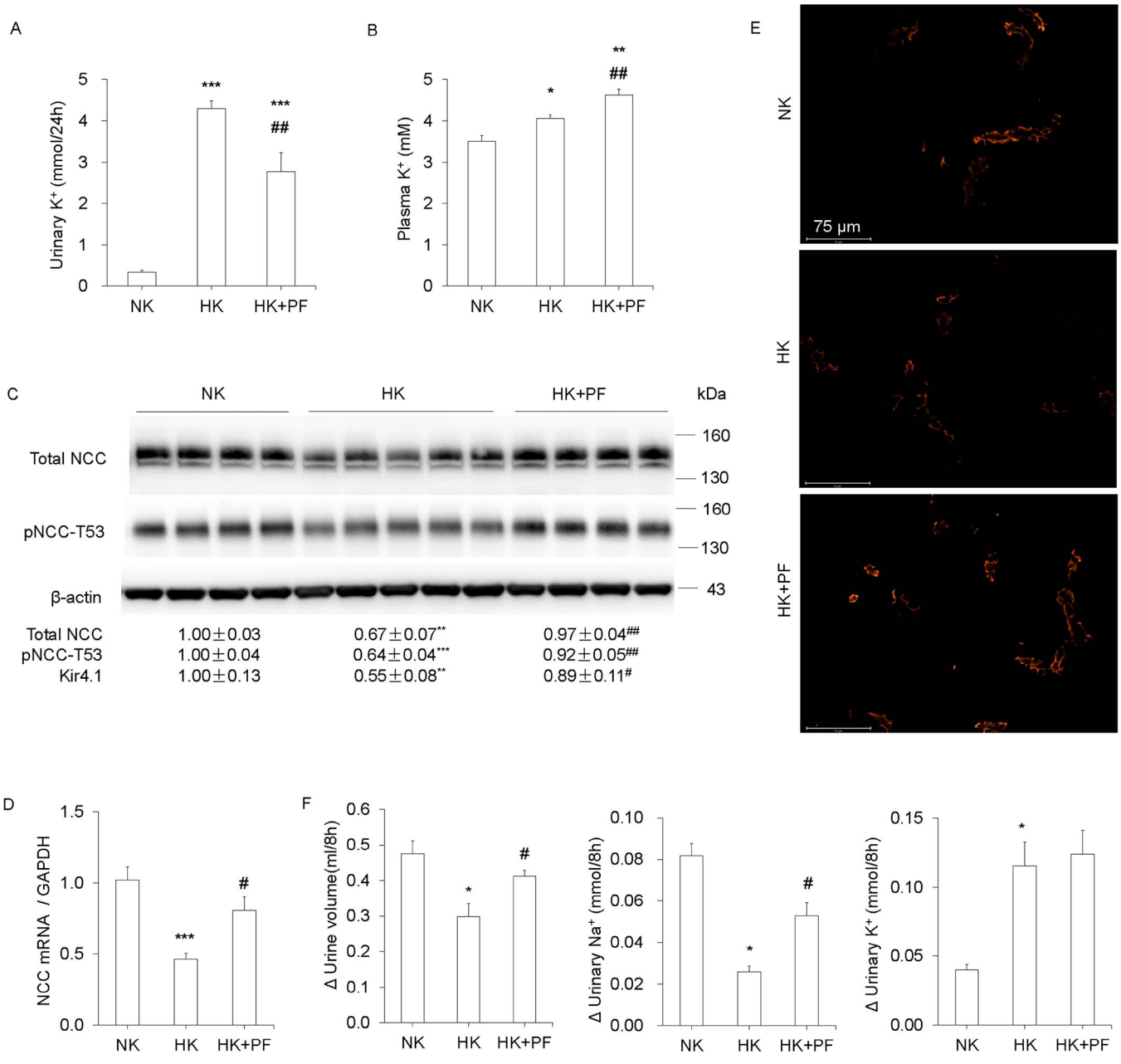

Figure 4.

Analysis of plasma K^+ concentration, urinary K^+ excretion, and pNCC in mice treated with NK, HK, or HK + PF. (A, B) Urinary K^+ excretion (A) and plasma K^+ concentration (B) in mice treated with NK, HK, or HK + PF. *p<0.05, **p<0.01, ***p<0.001 *vs.* NK; $^{\#\#}p$<0.01 *vs.* Floxed (ANOVA with the Bonferroni test). (C) Immunoblotting and densitometric analysis of NCC and pNCC-T53 in the renal cortex of normal mice treated with NK, HK, or HK + PF. A separated membrane was probed with anti-β-actin antibody as an internal control for equal loading of the samples. N = 5 per group. Data are mean ± SEM. **p<0.01, ***p<0.001 *vs.* NK; $^{\#}p$<0.05, $^{\#\#}p$<0.01 *vs.* HK (ANOVA with the Bonferroni test). (D) Representative immunofluorescence images of NCC and pNCC-T53 in DCT of normal mice

treated with NK, HK, or HK + PF. The images shown are representatives of 5 animals per group. (E) Effect of PF on *in vivo* NCC activity as reflected by rapid diuresis and natriuretic responses to HCTZ. NK, HK, and HK + PF mice were all subjected to a single dose of vehicle or HCTZ (10 mg/kg by gavage) treatment, followed by 8-h urine collection, and shown was the change in 8-h urinary Na^+ and K^+ excretion, determined by the delta value of 8-h urinary Na^+ or K^+ excretion of vehicle and HCTZ treatment. N = 10–20 per group. Data are mean ± SEM. *$p<0.05$ *vs.* NK; $^{\#}p<0.05$ *vs.* HK (ANOVA with the Bonferroni test).

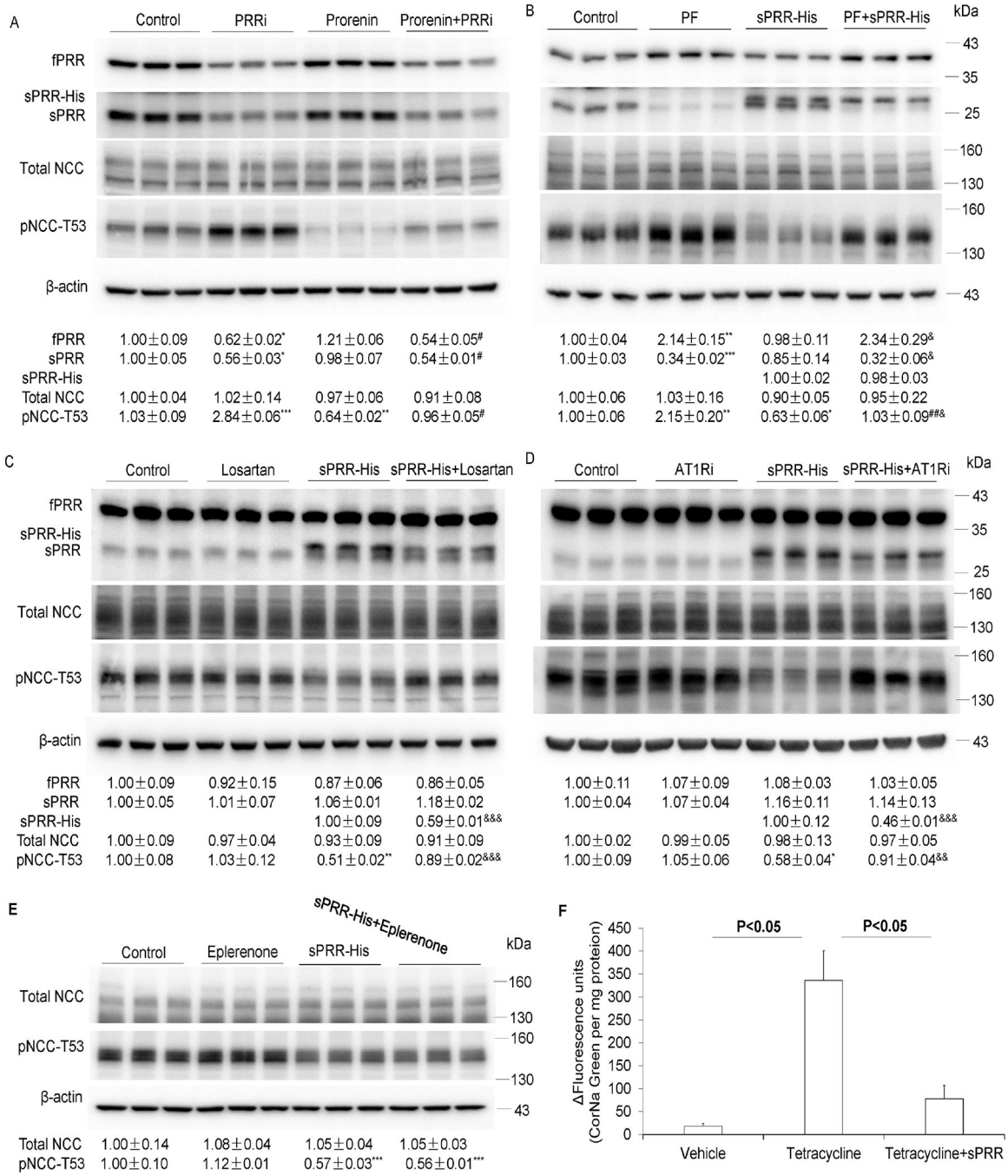

Figure 5.

Regulation of phosphorylation of NCC by sPRR in Flp-In T-REX 293 NCC cell line. (A) Validation of siRNA-mediated PRR knockdown and its effect on pNCC-T53 expression in Flp-In T-REX 293 NCC cell line. The cells were transfected with PRR siRNA and then treated with 100 nM prorenin for 24 h, then NCC and pNCC-T53 protein expression was analyzed by immunoblotting and densitometric analysis. PRR-probed membrane was stripped and re-probed with anti-β-actin antibody as an internal control for equal loading of the samples. N = 6 per group. Data are mean ± SEM. *p<0.05, **p<0.01, ***p<0.001

vs. Control; $^{\#}p<0.05$ *vs.* Prorenin (ANOVA with the Bonferroni test). (B) Effect of sPRR and PF429242 on expression of abundance of NCC and pNCC-T53. Flp-In T-REX 293 NCC cells were treated with 10 nM sPRR-His, or 5 μM PF429242, or 5 μM PF429242 in combination with 10 nM sPRR-His for 24 h, and the abundnaces of PRR, NCC, and pNCC-T53 were analyzed by immunoblotting and densitometric analysis. PRR-probed membrane was stripped and re-probed with anti-β-actin antibody. N = 6 per group. Data are mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, *vs.* Control; $^{\#\#}p < 0.01$ *vs.* PF; $^{\&}p < 0.05$ vs. sPRR-His (ANOVA with the Bonferroni test). (C, D) sPRR-His/AT1R signal regulates NCC-T53 phosphorylation. Cells were pretreated with 10 mM losartan (C) or transfected with AT1R siRNA (D), and then treated with 10 nM sPRR-His for 24 h. PRR, NCC, and pNCC-T53 protein expression was analyzed by immunoblotting and densitometric analysis. PRR-probed membrane was stripped and re-probed with β-actin as an internal control for equal loading of the samples. N = 6 per group. Data are mean ± SEM. $^{*}p<0.05$, $^{**}p<0.01$ *vs.* Control; $^{\&\&}p<0.01$, $^{\&\&\&}p<0.001$ vs. sPRR-His (ANOVA with the Bonferroni test). (E) Effect of mineralocorticoid receptor antagonist on the level of pNCC-T53. Cells were pretreated with 10 μM Eplerenone, and then treated with 10 nM sPRR-His for 24 h, then NCC and pNCC-T53 protein abundance was analyzed by immunoblotting and densitometric analysis. pNCC-T53-probed membrane was stripped and re-probed with anti-β-actin antibody. N = 3 per group. Data are mean ± SEM. $^{***}p < 0.001$, *vs.* Control (ANOVA with the Bonferroni test). (F) sPRR blocked sodium uptake via the inhibition of NCC in Flp-In T-REX 293 NCC cells. The values were normalized by protein content. N = 6 per group. Data are mean ± SEM. Statistical analysis was performed by using ANOVA with the Bonferroni test.