Sympathetic regulation of NCC in norepinephrine-evoked salt-sensitive hypertension in Sprague-Dawley rats

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Submitted 24 May 2019; accepted in final form 4 October 2019

INTRODUCTION

Dietary Na+ intake plays a significant role in blood pressure regulation, as increased Na+ retention is a well-established precursor of hypertension (15, 27, 44). Approximately 50% of hypertensive individuals and 25% of normotensive individuals exhibit salt sensitivity of blood pressure (1, 24), a phenomenon that can promote the development of salt-sensitive hypertension. Given that salt sensitivity is driven partly by a sympathoexcitatory response to dietary Na+ intake (7, 34) that drives Na+ retention, there is a pivotal role of the kidney in the pathophysiology of salt-sensitive hypertension. The Na+-Cl−-cotransporter (NCC), a thiazide-sensitive transporter predominantly found on the apical side of the distal convoluted tubule (DCT), plays a critical role in the fine tuning of Na+ reabsorption (13, 14, 32). Multiple recent studies have suggested a direct influence of the sympathetic nervous system release of norepinephrine (NE) on NCC expression and activity. In salt-resistant Sprague-Dawley rats, increased dietary salt intake suppresses sympathetic outflow and circulating NE levels (16, 17, 41) and persistently reduces NCC expression and activity (31, 43). NE infusion during normal salt (NS) intake appears to evoke a species-specific NCC response with no alteration in NCC expression in Sprague-Dawley rats (33, 43) versus an norepinephrine (NE)-evoked suppression of NCC, resulted in downregulation of WNK4, SPAK, and OxSR1, and abolished the salt-sensitive component of hypertension. In contrast, β-adrenoceptor antagonism attenuated NE-evoked hypertension independently of dietary Na+ intake and did not restore high salt-evoked suppression of NCC. These findings suggest that a selective, reversible, α1-adrenoceptor-gated WNK4-SPAK-OxSR1 NE-activated signaling pathway prevents dietary Na+-evoked NCC suppression, promoting the development and maintenance of salt-sensitive hypertension.

adrenoreceptors; blood pressure; norepinephrine; salt-sensitive hypertension; sodium-chloride cotransporter

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pathetic nervous system and subsequent changes in circulating NE levels, such as those seen in animal models of salt resistance or salt sensitivity, remain to be fully delineated.

Our prior study, conducted in male salt-resistant Sprague-Dawley rats, did not investigate the mechanisms or signal transduction pathways by which NE influences NCC activity during chronic alterations in dietary Na\(^+\) intake. As such, we hypothesized in the present study that in salt-sensitive hypertension, sympathoexcitation increases NCC activity via a NE-activated adrenoceptor-mediated signal transduction pathway that increases the expression and/or activity of NCC regulatory kinases. To test our hypothesis, we used NE-infused male Sprague-Dawley rats as a model of sympathetically mediated salt-sensitive hypertension (43). Here, we administered a chronic NE infusion during normal and high dietary salt intake and used coinusions of terazosin (\(\alpha_1\)-adrenoceptor antagonist) or propranolol (\(\beta\)-adrenoceptor antagonist) to provide novel mechanistic insights into the adrenergic signaling pathways regulating NCC activity and expression during the development and maintenance of salt-sensitive hypertension.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (9–12 wk old, Envigo, Indianapolis, IN) were randomly assigned to be fed a 21- or 42-day NS diet [0.6% NaCl, Teklad Global Diet no. 2918, Envigo Teklad, 18% protein, 5% crude fat, 5% fiber, total NaCl content: 0.6% (174 meq Na\(^+\)/kg) rodent diet or a high-salt (HS) diet [4% NaCl, Envigo Teklad Diet TD.03095, 19% protein, 5% crude fat, 3% fiber, total NaCl content: 4% (678 meq Na\(^+\)/kg)] and tap water ad libitum. All protocols were approved by the Boston University School of Medicine Institutional Animal Care and Use Committee under protocol no. 15241 and were approved by the Boston University School of Medicine Institutional Guide for the Care and Use of Laboratory Animals.

Surgical Procedures

Subcutaneous osmotic minipump implantation. Rats were anesthetized using sodium brevital (20 mg/kg ip). An osmotic minipump (2ML4, Alzet) was surgically implanted subcutaneously in the subscapular region.

Femoral vein, artery, and bladder cannulation. On days 21 or 42 of NS or HS intake, rats were anesthetized (sodium brevital, 20 mg/kg ip supplemented with 10 mg/kg iv, as required), and the left femoral vein, left femoral artery, and bladder were cannulated with PE-50 tubing to deliver intravenous infusions, measure arterial blood pressure, and collect urine, respectively (43). Rats received an infusion of isotonic saline (200 \(\mu\)L/min iv) during a 2-h recovery period to allow the animal to regain full consciousness and stable cardiovascular and renal excretory function (16, 41–43). Mean arterial pressure (MAP) was recorded continuously via the femoral artery cannula during the 1-h isotonic saline infusion period of the experimental period in each animal on days 21 or 42 of NS or HS intake.

Acute Experimental Protocols

The following protocols were performed consecutively in a single experimental period in each animal on days 21 or 42 of NS or HS intake.

Cardiovascular function. After the 2-h recovery period, baseline MAP was recorded continuously in conscious rats via the femoral artery cannula during the 1-h isotonic saline infusion period of the renal Na\(^+\) transporter activity protocol (see below). MAP values represent the average MAP during the entire 1-h period.

Renal Na\(^+\) transporter activity. Rats received an intravenous infusion for a 1-h control period [isotonic saline, 20 \(\mu\)L/min (4, 43)], a 1-h epithelial Na\(^+\) channel (ENaC) blockade period [amiloride, ENaC antagonist, 2 mg/kg bolus followed by 2 mg/kg \(\cdot\)1-h \(-1\) at 20 \(\mu\)L/min (4, 43)], and a 1-h NCC blockade period during which ENaC blockade was maintained [hydrochloroazide (HCTZ), NCC antagonist, 2 mg/kg bolus followed by 2 mg/kg \(\cdot\)1-h \(-1\) HCTZ + 2 mg/kg \(\cdot\)1-h \(-1\) amiloride at 20 \(\mu\)L/min (4, 43)]. The use of amiloride allows for the isolation of NCC’s contribution to urinary Na\(^+\) excretion. MAP readings were obtained during the 1-h control period. Throughout the protocol, urine was collected via the bladder cannula in 10-min increments to assess urine Na\(^+\) concentration. NCC activity was assessed as the peak natriuretic response [change in urinary excretion of Na\(^+\) (\(\Delta\)UNaV); in \(\mu\)eq/min] to HCTZ, calculated by subtracting average UNaV from the last two 10-min periods of ENaC blockade from maximum UNaV during NCC blockade (occurred within the first two 10-min periods of NCC blockade in all animals). ENaC activity was assessed as the peak natriuretic response (\(\Delta\)UNaV; in \(\mu\)eq/min) to amiloride, calculated by subtracting average UNaV from the last two 10-min periods of control from maximum UNaV during ENaC blockade (occurred within the first two 10-min periods of ENaC blockade in all animals).

Kidney mRNA Expression

Kidneys harvested from rats after decapitation and after completion of a 21-day NS or HS diet were immediately stored in RNA later RNA stabilization solution (catalog no. AM7021, Invitrogen) at −20°C. Approximately 100 mg of kidney cortex tissue were placed in 1 mL of QIAzol lysis reagent solution (catalog no. 79306, QIAGEN, Hilden, Germany) with a stainless steel bead (catalog no. 69989, QIAGEN) and homogenized using the QIAGEN TissueLyser II system at 30 Hz for 2 min (50). Samples were then left at room temperature for 5 min before being treated with 100 \(\mu\)L of gDNA Eliminator solution (catalog no. 1062831, QIAGEN). To extract nucleic acids, 200 \(\mu\)L chloroform was added to each sample before being centrifuged at 17,000 g for 15 min at 4°C. Approximately 600 \(\mu\)L of the top aqueous layer were taken for mRNA purification using the QIACube (QIAGEN) with RNase Plus Universal Kit reagents and protocol (catalog no. 73404, QIAGEN). Purified mRNA from each sample was converted to cDNA with a High Capacity RNA-to-cDNA kit (catalog no. 4387406, Applied Biosystems). Real-time PCR was performed using a ViiA7 thermal cycler (Applied Biosystems), RT\(^2\) SYBR Green ROX qPCR Mastermix (catalog no. 330529, QIAGEN), and a custom Rat RT\(^2\) Profiler PCR array with propriety primer sequences (catalog no. CAPR11754, QIAGEN). mRNA expression for all target genes was normalized to the expression of the following four housekeeping genes: \(\beta\)-actin (ACTB), hypoxanthine phosphoribosyltransferase-1 (HPRT1), lactate dehydrogenase A (LDHA), and
ribosomal protein lateral stalk subunit P1 (RPLP1). The ΔΔCT method (50) (where C_{T} is threshold cycle) was used to calculate mRNA expression changes between groups. Readouts are expressed as fold changes of NS + saline target gene expression. Analysis was carried out for each individual sample, as mRNA was not pooled.

**Kidney Protein Extract Preparation**

Kidneys harvested from rats after decapitation and after completion of the acute experiments were immediately stored at −80°C. Approximately 200 mg of kidney cortex tissue were homogenized on ice using a hand pestle in a homogenizing buffer containing Halt protease inhibitor cocktail (catalog no. 78429, Thermo Scientific) and PhosSTOP phosphatase inhibitor (catalog no. 04906845001, Roche) (43). The homogenate was then centrifuged at 4,000 rpm for 10 min at 4°C (43). The supernatant was collected and further centrifuged at 17,000 g for 1 h at 4°C. The pellet was then resuspended using 400 μL of 1× cell lysis buffer (43). A BCA assay was used to determine protein content. Protein extract preparations were stored at −80°C for use in immunoblot experiments.

**Immunoblot Analysis**

Kidney cortex protein extracts were loaded at 20 μg protein/lane. Nitrocellulose membranes (catalog no. 10600096, GE) were blocked in 5% blocking grade blocker (catalog no. 170-6404, Bio-Rad) for 1 h and probed overnight at 4°C with primary antibodies in 0.1% PBS-Tween 20. Membranes were washed with 0.1% PBS-Tween 20 and incubated for 2 h with secondary antibodies in 0.1% PBS-Tween 20 at room temperature. Membranes were visualized using chemiluminescence (SuperSignal West Pico Plus, catalog no. 34580, Thermo Scientific). Quantitative analysis was performed using ImageJ (version 1.52, National Institutes of Health), and protein expression was normalized to total protein using Coomassie staining (Bio-Safe, catalog no. 1610786, Bio-Rad) (11). The antibodies and dilutions used are shown in Table 1.

**Analytic Techniques**

Urine volume was determined gravimetrically, assuming 1 g = 1 mL. Urine Na⁺ and K⁺ concentrations were assessed using flame photometry (model 943, Instrumentation Laboratories) (16, 41–43).

**Statistical Analysis**

Data are shown as means ± SE. Comparisons were made between NS and HS dietary salt intake within groups using a two-tailed Student’s t test, two-way ANOVA was used to assess differences between groups, and Tukey’s post hoc test was used to evaluate variations among groups. Statistical analysis was carried out using GraphPad Prism (version 7). Statistical significance was defined as P < 0.05.

### Table 1. Antibody dilutions used for immunoblotting studies

| Antibodies                        | Dilution | Source                                           |
|-----------------------------------|----------|--------------------------------------------------|
| NCC                               | 1:1,000 (43) | Catalog no. AB3553, Millipore, Billerica, MA    |
| Phosphorylated NCC (Thr53)        | 1:1,000 (9)    | Catalog no. p1311-53, Phosphosolutions, Aurora, CO |
| WNK1                              | 1:200 (48)     | Catalog no. 28897, Santa Cruz Biotecnology, Dallas, TX |
| WNK4                              | 1:1,000       | Catalog no. NB600-2848S, Novus Biologicals, Centennial, CO |
| OxSR1                             | 1:2,000       | Catalog no. ab125468, Abcam, Cambridge, MA      |
| SPAK                              | 1:500        | Catalog no. ab79045, Abcam                       |
| Anti-phospho-SPAK (Ser73S)/phospho-OSR1 (Ser125S) | 1:500 | Catalog no. 07-2273, Millipore Sigma, Burlington, MA |
| Secondary antibody                | 1:2,000 (43)     | Catalog no. ab16284, Abcam                      |

NCC, Na⁺-Cl⁻ cotransporter; WNK, with no lysine kinase; OxSR1, oxidative stress response 1; SPAK, STE20/SPS1-related proline-alanine-rich protein kinase.

**RESULTS**

**Impact of Chronic NE Infusion on Blood Pressure and NCC Regulation**

In saline-infused rats, HS intake did not alter blood pressure or basal Na⁺ or K⁺ excretion during the renal Na⁺ transporter assay and promoted the suppression of NCC activity, as assessed by the natriuretic response to the NCC antagonist HCTZ (NCC activity: 9.8 ± 0.5 μeq/min in the NS + saline group vs. 6.5 ± 0.4 μeq/min in the HS + saline group, P < 0.05; Fig. 1, A and B, and Table 2). Dietary Na⁺-evoked suppression of NCC activity in saline-infused rats was paralleled by reductions in NCC mRNA, protein expression, and phosphorylation at Thr53 (Fig. 1, C and D, Fig. 2, and Fig. 3A).

NE-infused rats fed a NS diet exhibited an increase in blood pressure (MAP: 149 ± 4 mmHg in the NS + NE group vs. 122 ± 2 mmHg in the NS + saline group, P < 0.05) that occurred independently of alterations in NCC activity, expression, or phosphorylation (Fig. 1, A–D). A 21-day HS diet exacerbated hypertension (MAP: 169 ± 5 mmHg) in NE-infused rats, indicating the development of salt-sensitive hypertension (Fig. 1A). Critically, NE-infused rats failed to suppress NCC in vivo activity during HS intake (Fig. 1B). Mirroring these findings, NE-infused rats failed to suppress NCC mRNA and total protein expression and NCC phosphorylation at Thr53 during HS intake (Fig. 1, C and D, Fig. 2, and Fig. 3A).

**Impact of Chronic NE Infusion on NCC Regulatory Kinases and Adrenoceptor Subtypes**

Total WNK1 and OxSR1 protein expression was suppressed during HS intake in saline-infused rats (fold change in WNK1 expression: 1 ± 0.08 in the NS + saline group vs. 0.44 ± 0.08 in the HS + saline group; fold change in OxSR1 expression: 1 ± 0.07 in the NS + saline group vs. 0.48 ± 0.13 in the HS + saline group, P < 0.05), whereas WNK4 expression increased (fold change in WNK4 expression: 1 ± 0.19 in the NS + saline group vs. 2.7 ± 0.38 in the HS + saline group, P < 0.05; Figs. 1, E, F, and H, and 2). SPAK expression was unaffected by dietary salt intake in saline-infused rats, whereas SPAK/OxSR1 phosphorylation showed a decreasing trend with HS intake (Figs. 1, G and I, and 2). In contrast, impaired dietary Na⁺-evoked NCC suppression in NE-infused rats was accompanied by a failure to suppress WNK1 or OxSR1 protein expression during HS intake (Figs. 1, E and H, and 2). WNK4
and SPAK expression were also unchanged in NE-infused rats (Figs. 1, F and G, and 2). Critically, SPAK/OxSR1 phosphorylation exhibited an increasing trend in NE-infused rats on a HS diet in contrast to the lowering trend in saline-infused rats on a HS diet (Figs. 1 I and 2).

At the mRNA level, WNK1 expression was increased by NE infusion during NS intake (fold change in mRNA expression: 1.49 ± 0.12 in the NS + NE group vs. 1 in the NS + saline group, \( P < 0.05 \)) and remained elevated during HS intake (1.74 ± 0.15 in the HS + NE group; Fig. 3A). In contrast, WNK3 and WNK4 mRNA expression were not influenced by treatment or diet (Fig. 3A). In NE-infused rats maintained on a 21-day HS diet, OxSR1 mRNA expression was elevated compared with saline-infused rats during HS intake (fold change in OxSR1 mRNA expression: 1.76 ± 0.14 in the HS + NE group vs. 1.12 ± 0.12 in the HS + saline group, \( P < 0.05 \)), and SPAK mRNA expression was increased compared with both saline-infused rats during HS intake and NE-infused rats during NS intake (fold change in SPAK mRNA expression: 1.98 ± 0.26 in the HS + NE group vs. 1.08 ± 0.18 in the
Impact of Chronic $\alpha_1$-Adrenoceptor Antagonism on the Development of NE-Evoked Salt-Sensitive Hypertension

Chronic $\alpha_1$-adrenoceptor antagonism using terazosin infusion alone did not alter blood pressure or basal Na\(^+\) or K\(^+\) excretion during the renal Na\(^+\) transporter assay or in vivo NCC activity in saline-infused rats during NS intake (Fig. 4A and B, and Table 2). Moreover, terazosin-infused rats maintained baseline blood pressure and retained the ability to suppress NCC activity during the 21-day HS diet (NCC activity: 10.6 $\pm$ 0.6 $\mu$eq/min in the NS + saline + terazosin group vs. 6.8 $\pm$ 0.4 $\mu$eq/min in the HS + saline + terazosin group, $P < 0.05$; Fig. 4, A and B). Significantly, whereas $\alpha_1$-adrenoceptor blockade did not alter blood pressure or NCC activity in NE-infused rats during NS intake, terazosin prevented the development of the salt-sensitive component of hypertension in NE-infused rats (MAP: 172 $\pm$ 5 mmHg in the HS + NE group vs. 147 $\pm$ 3.6 mmHg in the HS + NE + terazosin group, $P < 0.05$) and restored dietary Na\(^+\)-evoked suppression of in vivo NCC activity (NCC activity: 10.7 $\pm$ 0.8 $\mu$eq/min in the NS + NE + terazosin group vs. 6.1 $\pm$ 1.2 $\mu$eq/min in the HS + NE + terazosin group, $P < 0.05$; Fig. 4, A and B). In all rats, selective $\alpha_1$-adrenoceptor antagonism was confirmed as the loss of a pressor response to phenylephrine, whereas the tachycardic response to isoproterenol remained intact (Fig. 4, C and D).

Impact of Chronic $\beta$-Adrenoceptor Antagonism on the Development of NE-Evoked Salt-Sensitive Hypertension

Chronic $\beta$-adrenoceptor antagonism using propranolol infusion alone did not alter baseline blood pressure or basal Na\(^+\) or K\(^+\) excretion during the renal Na\(^+\) transporter assay or NCC activity during NS or HS intake in saline-infused rats (Fig. 5, A and B, and Table 2). In NE-infused rats, propranolol attenuated the development of NE-evoked hypertension (MAP: 151 $\pm$ 5 mmHg in the NS + NE group vs. 136 $\pm$ 4 mmHg in the NS + NE + propranolol group, $P < 0.05$) and abolished the salt sensitivity of blood pressure (MAP: 171 $\pm$ 5 mmHg in

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| Table 2. Basal Na\(^+\) and K\(^+\) excretion |
|---------------------------------------------|
| Subcutaneous | Saline + terazosin | Subcutaneous | NE + terazosin |
| Normal | 2.55 $\pm$ 0.36 | 2.26 $\pm$ 0.32 | 2.23 $\pm$ 0.45 |
| HS | 2.03 $\pm$ 0.45 | 2.31 $\pm$ 0.47 | 2.43 $\pm$ 0.36 |

Values are expressed as means $\pm$ SE; $n = 6$ rats/group. Basal Na\(^+\) and K\(^+\) excretion were determined during the control period of the renal Na\(^+\) transporter assay or in vivo NCC transporter assay or NCC activity assay in conscious 3-mo-old male Sprague-Dawley rats that received a subcutaneous infusion of saline or norepinephrine (NE) alone or in combination with terazosin or propranolol during 21-day normal-salt (0.6% NaCl) or high-salt (4% NaCl) diets.

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the HS + NE group vs. 135 ± 5 mmHg in the HS + NE + propranolol group, \( P < 0.05 \); Fig. 5A). Propranolol, however, did not alter NCC activity during either diet, suggesting that chronic NE-mediated NCC regulation occurs independently of \( \beta \)-adrenoceptor signaling (Fig. 5B). In all rats, selective \( \beta \)-adrenoceptor antagonism was confirmed as the loss of a tachycardic response to isoproterenol, whereas the pressor response to phenylephrine remained intact (Fig. 5, C and D).

![Representative immunoblots for saline-and norepinephrine (NE)-infused Sprague-Dawley (SD) rats.](image)

![Impact of chronic norepinephrine (NE) infusion on mRNA expression of the Na⁺-Cl⁻ cotransporter (NCC), its regulatory kinases, and adrenoceptor subtypes.](image)

**Fig. 2.** Representative immunoblots for saline- and norepinephrine (NE)-infused Sprague-Dawley (SD) rats. Immunoblots are shown for NaCl cotransporter (NCC), phosphorylated (pNCC (Thr\(^5\)) (pNCCT53), with no lysine kinase (WNK)1, WNK4, STE20/SPS1-related proline-alanine-rich kinase (SPAK), oxidative stress response 1 (OxSR1), and pSPAK/OxSR1. Coomassie-stained gels are shown for total protein in 3-mo-old male SD rats that received a subcutaneous infusion of saline or NE during a 21-day normal-salt (NS; 0.6% NaCl) or high-salt (HS; 4% NaCl) diet.

**Fig. 3.** Impact of chronic norepinephrine (NE) infusion on mRNA expression of the Na⁺-Cl⁻ cotransporter (NCC), its regulatory kinases, and adrenoceptor subtypes. A and B: renal cortex mRNA expression of NCC and its regulatory kinases (A) and adrenoceptor subtypes (B) in 3-mo-old male Sprague-Dawley (SD) rats that received a subcutaneous infusion of saline or NE during 21-day normal-salt (NS; 0.6% NaCl) or high-salt (HS; 4% NaCl) diet. \( n = 6 \) rats/group. Differences were determined using two-way ANOVA and a Tukey’s post hoc test. \(* P < 0.05\) vs. the respective NS group; \( \tau P < 0.05\) vs. the respective saline-infused group.
Impact of Chronic α1- and β-Adrenoceptor Antagonism on NCC Regulatory Kinases

Significantly, the restoration of dietary Na⁺-evoked suppression of NCC activity by α1-adrenoceptor blockade was paralleled by restored suppression of NCC expression (fold change in NCC expression: 1 ± 0.09 in the NS + NE + terazosin group vs. 0.63 ± 0.07 in the HS + NE + terazosin group, P < 0.05), NCC phosphorylation (fold change in NCC phosphorylation expression: 1 ± 0.12 in the NS + NE + terazosin group vs. 0.34 ± 0.05 in the HS + NE + terazosin group, P < 0.05), and OxSR1 expression during HS intake (fold change in OxSR1 expression: 1 ± 0.18 in the NS + NE + terazosin group vs. 0.41 ± 0.06 in the HS + NE + terazosin group, P < 0.05; note that one sample value was discarded as an outlier determined by ROUT analysis; Figs. 6, A, B, and F, and 7). α1-Adrenoceptor blockade failed to restore dietary Na⁺-evoked suppression of WNK1 expression (Figs. 6C and 7). In contrast to saline-infused rats, α1-adrenoceptor blockade resulted in a decrease rather than an increase in WNK4 expression (fold change in WNK4 expression: 1 ± 0.08 in the NS + NE + terazosin group vs. 0.45 ± 0.05 in the HS + NE + terazosin group, P < 0.05; Figs. 6D and 7) and also resulted in a decrease in SPAK expression (fold change in SPAK expression: 1 ± 0.11 in the NS + NE + terazosin group vs. 0.43 ± 0.01 in the HS + NE + terazosin group, P < 0.05; Figs. 6E and 7). Phosphorylated SPAK/OxSR1 expression was unchanged with α1-adrenoceptor blockade (Figs. 6G and 7).

Propranolol infusion prevented NE-evoked hypertension but did not alter NCC regulation in NE-infused rats and also failed to restore the suppression of NCC expression and phosphorylation during HS intake (Figs. 6, A and B, and 7). Furthermore, whereas β-adrenoceptor blockade restored suppression of OxSR1 expression during HS intake in NE-infused rats (fold change in OxSR1 expression: 1 ± 0.06 in the NS + NE + propranolol group vs. 0.52 ± 0.13 in the HS + NE + propranolol group, P < 0.05), dietary Na⁺-evoked suppression of WNK1 expression remained impaired (Figs. 6, C and F, and 7). SPAK expression was unchanged, whereas phosphorylated SPAK/OxSR1 significantly increased, with β-adrenoceptor blockade (fold change in phosphorylated SPAK/OxSR1 expression: 1 ± 0.29 in the NS + NE + propranolol group vs. 3.8 ± 0.43 in the HS + NE + propranolol group, P < 0.05; Figs. 6, E and G, and 7).

Impact of Chronic α1-Adrenoceptor Antagonism on Established NE-Evoked Salt-Sensitive Hypertension

A 42-day HS diet did not alter blood pressure and promoted the suppression of NCC activity in saline-infused rats (NCC activity: 10 ± 0.9 μeq/min in the NS + saline group vs. 6.7 ± 0.6 μeq/min in the HS + saline group, P < 0.05; Fig. 8, A and B). A 42-day NE infusion evoked hypertension (MAP: 142 ± 2 mmHg in the NS + NE group) but had no impact on NCC activity during NS intake and promoted the development of salt-sensitive hypertension (MAP: 164 ± 4 mmHg in the HS + NE vs. 142 ± 2 mmHg in the NS + NE group, P < 0.05) accompanied by impaired dietary Na⁺-evoked NCC suppression (Fig. 8, A and B). Terazosin infusion did not alter blood pressure or NCC activity in NE-infused rats maintained on a NS diet (Fig. 8, A and B). Critically, however, α1-adrenoceptor blockade reversed the salt-sensitive component of established NE-evoked salt-sensitive hypertension (MAP: 139 ± 3 mmHg in the HS + NE + terazosin group vs. 164 ± 4 in the HS + NE group, P < 0.05) and restored dietary Na⁺-evoked suppression of NCC activity (NCC activity: 10.5 ± 0.9 μeq/min in...
the NS + NE + terazosin group vs. 6.3 ± 0.06 µeq/min in the HS + NE + terazosin group, P < 0.05; Fig. 8, A and B).

**NE-Evoked Salt-Sensitive Hypertension Is Independent of ENaC Activity**

In vivo ENaC activity was assessed as the natriuretic response to the ENaC antagonist amiloride during the renal transporter assay. As previously shown (43), saline-infused rats suppressed ENaC activity during a HS diet (ENaC activity: 8.7 ± 0.6 µeq/min in the NS + saline group vs. 4.1 ± 0.4 µeq/min in the HS + saline group, P < 0.05; Fig. 9). NE-infused rats maintained dietary evoked suppression of ENaC activity (ENaC activity: 9.8 ± 0.6 µeq/min in the NS + NE group vs. 3.8 ± 0.7 µeq/min in the HS + NE group, P < 0.05; Fig. 9). Moreover, neither α1-adrenoceptor antagonism nor β-adrenoceptor antagonism prevented dietary evoked suppression of ENaC activity in saline- or NE-infused rats (ENaC activity: 8.9 ± 0.6 µeq/min in the NS + saline + terazosin group vs. 4.3 ± 0.7 µeq/min in the HS + saline + terazosin group, P < 0.05; 9.4 ± 0.8 µeq/min in the NS + NE + terazosin group vs. 4.0 ± 0.6 µeq/min in the HS + NE + terazosin group, P < 0.05; 9.2 ± 0.8 µeq/min in the NS + saline + propranolol group vs. 3.6 ± 0.8 µeq/min in the HS + saline + propranolol group, P < 0.05; 8.9 ± 0.7 µeq/min in the NS + NE + propranolol group vs. 4.1 ± 0.6 µeq/min in the HS + NE + propranolol group), P < 0.05; Fig. 9).

**DISCUSSION**

The present study was designed to delineate the adrenergic signaling pathways that promote NCC activity in NE-evoked salt-sensitive hypertension. Previous studies have demonstrated that exogenous NE drives increased NCC activity and salt-sensitive hypertension in Sprague-Dawley rats (43), which are classically salt resistant (16, 17), and that salt-sensitive animal models exhibit excess sympathetic outflow during HS intake that could promote NCC activation (25, 38). Here, we used α1- and β-adrenoceptor antagonism to provide new mechanistic insights into a selective α1-adrenoceptor-gated WNK/SPAK/OxSR1 signaling pathway that we hypothesized prevents the suppression of NCC expression, phosphorylation, and activity during the development and maintenance of NE-evoked salt-sensitive hypertension in male Sprague-Dawley rats.

Our present observations are consistent with previous studies that reported dietary Na+ -evoked suppression of NCC activity in normotensive Sprague-Dawley rats (43, 46) and linking sympathetic stimulation of the NCC with salt-sensitive hypertension in Sprague-Dawley rats and other rodent models (25, 38, 43). It should be noted that basal Na+ and K+ excretion during the renal Na+ transporter assay was unchanged in all rats and that the suppression of ENaC activity in response to HS intake was observed in all treatment groups (Fig. 9 and Table 2) and is consistent with our previous study (43). These data suggest that ENaC activity does not contribute to the development of NE-evoked hypertension or NE-evoked salt-sensitive hypertension in Sprague-Dawley rats. During NS intake, NE infusion promoted an increase in blood pressure that occurred independently of alterations in NCC activity and regulation and likely reflects the effects of NE in other organ systems (e.g., the vasculature and heart) and other receptor and signal transduction pathways beyond NCC. Studies in mice have reported a NE-evoked increase in NCC expression during NS intake (25, 38). Our finding that NE alone does not alter in vivo NCC activity is consistent with previous studies in Sprague-Dawley rats (33, 43) and suggests there may be species differences in NE-mediated NCC regulation.
Fig. 6. Impact of chronic \(\alpha_1\)- and \(\beta\)-adrenoceptor antagonism on the Na\(^{+}\)-Cl\(^{-}\) cotransporter (NCC) and its regulatory kinases. A–G: renal cortex total NCC expression (A), phosphorylated (pNCC (Thr\(^53\)) (pNCC/T53; B), with no lysine kinase (WNK)1 expression (C), WNK4 expression (D), STE20/SPS1-related proline-alanine-rich kinase (SPAK) expression (E), oxidative stress response 1 (OxSR1) expression (F), and SPAK/OxSR1 phosphorylation (pSPAK/OxSR1; G) in 3-mo-old male Sprague-Dawley (SD) rats that received a subcutaneous infusion of saline, norepinephrine (NE), NE + terazosin, or NE + propranolol during 21-day normal-salt (NS; 0.6% NaCl) or high-salt (HS; 4% NaCl) diet. \(n = 5/6\) rats/group. Data for subcutaneous saline and subcutaneous NE groups are replicated from Fig. 1 for clarity. Protein expression is shown as fold changes, with NS target protein expression set to 1 for each treatment. Differences between NS and HS target protein expression within each treatment group were determined using a Student’s \(t\) test. *\(P < 0.05\) vs. the respective NS group.
Numerous studies have supported WNK1 and WNK4 as regulators of NCC activity; however, the exact mechanism by which these kinases regulate NCC is unclear and remains controversial. WNK1 and WNK4 are able to stimulate NCC activity via SPAK and OxSR1 (23, 30). Adding to the ambiguity, the WNK1 gene can be expressed as two isoforms: a full-length isoform, L-WNK1, and a shorter, kinase-deficient isoform, kidney-specific WNK1 (KS-WNK1) (35). A previous study (35) in *Xenopus* oocytes suggested that KS-WNK1 forms protein complexes with L-WNK1, thereby inhibiting its ability to influence NCC activity; however, a recent study (2) has shown that KS-WNK1 can stimulate NCC activity. In the context of salt-sensitive hypertension, an initial study (25) assessing NCC regulation suggested that NE activates a $\beta_2$-adrenergic pathway involving WNK4 but not WNK1. Subsequent studies failed to replicate the role of WNK4 but did not assess WNK1 (38, 39), and the potential role of WNK1 in NE-mediated NCC activation remains largely unaddressed.

**Fig. 7.** Representative immunoblots for saline or norepinephrine (NE)-infused Sprague-Dawley (SD) rats treated with $\alpha_1$- or $\beta$-adrenoceptor antagonists. Immunoblots are shown for Na$^+$/Cl$^-$ cotransporter (NCC), phosphorylated (pNCC) (Thr$^{53}$) (pNCCT53), with no lysine kinase (WNK)1, WNK4, STE20/SPS1-related proline-alanine-rich kinase (SPAK), oxidative stress response 1 (OxSR1), pSPAK/OxSR1. Coomassie-stained gels are shown for total protein in SD rats that received a subcutaneous infusion of NE in combination with terazosin or propranolol during 21-day normal-salt (NS; 0.6% NaCl) or high-salt (HS; 4% NaCl) diet. n = 6 rats/group.

**Fig. 8.** Impact of chronic $\alpha_1$-adrenoceptor antagonism on established norepinephrine (NE)-evoked salt-sensitive hypertension. A and B: mean arterial pressure (MAP; in mmHg; A) and peak natriuretic response [change in urinary excretion of Na$^+$ (ΔUNaV); μeq/min] to intravenous hydrochlorothiazide (HCTZ; 2 mg/kg bolus, 2 mg·kg$^{-1}$·h$^{-1}$ infusion; B) in 3-mo-old male Sprague-Dawley (SD) rats on a 42-day normal-salt (NS; 0.6% NaCl) or high-salt (HS; 4% NaCl) diet that received a subcutaneous infusion of saline (42 days; black bars), NE alone (42 days; red bars), or NE alone for 21 days followed by NE + terazosin for 21 days (blue bars). n = 6 rats/group. Differences were determined using two-way ANOVA and a Tukey’s post hoc test. *P < 0.05 vs. the respective NS group; †P < 0.05 vs. the respective NE-infused group.
In the present study, we observed that total WNK1 and OxSR1 protein expression were suppressed, whereas WNK4 protein expression was increased, during HS intake in salt-resistant saline-infused rats but not in NE-infused rats exhibiting salt-sensitive hypertension. These changes paralleled impairments in dietary Na\(^+\)-evoked suppression of NCC activity, expression, and phosphorylation, providing strong evidence for roles of WNK1 and WNK4 in NE-mediated NCC regulation in this model of salt sensitivity. A limitation of our study, unlike a previous study performed in cell lines (6), is that antibodies directed specifically against L-WNK1 or KS-WNK1 are not presently available. Moreover, the WNK1 antibody used in this study targets the carboxyl terminus of WNK1 and cannot theoretically recognize both WNK isoforms (but not distinguish between them) and results in bands at ~100, 150, and 250 kDa (Figs. 2 and 7). It should be noted that L-WNK1 encodes a protein with a molecular mass of 250 kDa, whereas KS-WNK1 protein has an approximate molecular mass of 230 kDa. We therefore chose to assess total WNK1 expression in this study as the signal at 250 kDa, as it likely reflects both L-WNK1 and KS-WNK1 expression.

Our findings are highly suggestive of a WNK1/4 interaction that contributes to the ability of male Sprague-Dawley rats to suppress NCC activity during HS intake. We speculate that WNK1 is functioning as a positive regulator of NCC activity that is suppressed during HS intake, whereas WNK4 is functioning as a negative regulator of NCC that is increased during HS intake. These observed changes in the WNK1/4 dynamic may contribute to dietary-evoked suppression of NCC activity/expression. Despite the unclear relationship between WNK1 and WNK4, a previous study (5) has suggested that WNK4 may directly inhibit WNK1’s ability to stimulate NCC activity and indirectly inhibit WNK1’s stimulation of the NCC by preventing WNK1’s capacity to interact with OxSR1, thereby preventing downstream activation of NCC. Interestingly, the observed increase in WNK4 expression corresponded to a decrease in both WNK1 and OxSR1 expression, which may contribute to the suppression of NCC activity/expression in saline-infused rats. WNK4’s speculative role as a negative regulator of NCC is in contrast to studies that suggested that WNK4 is an activator of NCC in humans (47) and to data from WNK4 knockout mouse models where WNK4 acts as a positive regulator of NCC (36). Moreover, we acknowledge that the changes in WNK1/4 abundance in response to dietary salt intake observed in kidney cortex homogenates in this study may not reflect the expression of these kinases specifically in the DCT where NCC is expressed. Therefore, future studies are needed to address DCT-specific expression of the WNKs and their effects on NCC regulation.

WNK4 mRNA expression was unchanged in saline- and NE-infused rats irrespective of dietary salt intake level. These findings do not correlate with WNK4 protein expression in saline-infused rats and highlight the well-documented disconnect between mRNA levels and subsequent protein expression. Moreover, these results partially contrast with a prior study (20) in which Sprague-Dawley rats fed a HS diet showed an increase in WNK4 mRNA and protein expression compared with rats fed a NS diet. These discrepancies may be explained by the difference in quantitative PCR methodology, as our study assessed mRNA expression using a verified quantitative PCR array in which expression was normalized to four housekeeping genes, whereas the contrasting study used custom primers and a single housekeeping gene. As such, further studies are needed to address the role of WNK4 mRNA in NE-mediated NCC regulation.

Importantly, SPAK and OxSR1 phosphorylation, which occurs downstream of WNK1 or WNK4 and reflects activation of SPAK and OxSR1 (23), leads to NCC phosphorylation and activation (29) and shows a lowering trend in saline-infused rats and an increasing trend in NE-infused rats during HS intake compared with NS intake. This alteration in SPAK/OxSR1 phosphorylation may contribute to NE-evoked salt sensitivity. Given the lack of antibodies that specifically target either phosphorylated SPAK or phosphorylated OxSR1, we acknowledge that the independent activity or contributions of SPAK or OxSR1 cannot be assessed, and further studies are needed to distinguish the importance of each kinase in this signaling pathway.

Moreover, whereas previous studies have suggested a relatively minor contribution of OxSR1 in NCC regulation, our finding that high dietary salt intake evokes a decrease in total OxSR1 expression in saline-infused rats that is lost with NE infusion is consistent with an in vivo study (38) in which kidney-specific deletion of OxSR1 severely attenuated NE-
evoked NCC phosphorylation 30 min post-NE stimulation. These data suggest a potentially greater role of OxSR1 in long-term NCC regulation than has been previously reported. However, it should be noted that the DCT-specific expression of SPAK or OxSR1 was not addressed in this study, and the relative contributions of each kinase in the DCT remain to be determined in future studies. Additionally, NE prevented dietary evoked suppression of NCC phosphorylation at Thr53, which may contribute to observed increases in NCC activity in vivo. However, the role of phosphorylation at other residues relevant to NCC activation, and the role of other posttranslational modifications that modulate NCC activity, including ubiquitination (3), require further study that is beyond the scope of this investigation.

Antagonism of α1D- or β1-adrenoceptors did not alter blood pressure or NCC activity in saline-infused rats regardless of dietary Na+ intake, suggesting that blood pressure and NCC activity are determined by other physiological factors under normotensive conditions. In NE-infused animals placed on the HS diet, the development of the salt-sensitive component of hypertension was abolished by α1D-adrenoceptor blockade, which we speculate reflects the restoration of dietary Na+-evoked suppression of NCC activity, expression, and phosphorylation. Mechanistically, α1D-adrenoceptor blockade restores dietary Na+-evoked suppression of the regulatory kinase OxSR1 and also results in decreased WNK4 and SPAK expression. It is likely that NE upsets the balance of WNK1/ WNK4 expression that contributes to the ability of saline-infused rats to downregulate NCC activity/expression. The observed decrease with α1D-adrenoceptor antagonism in WNK4 expression may lead to the decrease in NCC activity/expression, suggesting that in NE-infused rats, WNK4 rather than WNK1 may play a greater role regulating NCC activity and, interestingly, act as a positive regulator of NCC. Our speculated role for WNK4 in NCC regulation is somewhat supported by a previous study (36) where WNK4 knockout failed to prevent the suppression of NCC in mice fed a HS diet. Likewise, the suppression of SPAK and OxSR1 expression coupled with the ability of α1D-adrenoceptor blockade to prevent the increasing trend in SPAK/OxSR1 phosphorylation observed with NE-infused rats may contribute to reduced NCC activity/phosphorylation.

In contrast, antagonism of β-adrenoceptors attenuated NE-evoked hypertension independently of dietary Na+ intake but failed to restore dietary Na+-evoked suppression of NCC activity or expression. This suggests that a significant component of NE-evoked hypertension is mediated via β-adrenoceptor activation independently of the regulation of NCC. Antagonism of β-adrenoceptors failed to restore dietary Na+-evoked suppression of the expression of the regulatory kinase WNK1. Critically, restoration of dietary Na+-evoked suppression of OxSR1 by β-adrenoceptor antagonism in the absence of the reduction of NCC activity supports a model in which SPAK plays a more significant role in NCC regulation in the context of salt-sensitive hypertension. These data suggest a potential critical and selective role for α1D-adrenergic signaling pathways in NE-evoked NCC dysregulation and salt-sensitive hypertension that conflicts somewhat with previous studies.

In a study of NE-infused C57B1/6J mice (25), antagonism of β-adrenoceptors, but not α1D-adrenoceptors, attenuated NE-induced downregulation of WNK4 mRNA and upregulation of NCC protein expression. In opposition, we observed that antagonism of α1D-adrenoceptors resulted in decreased WNK4 protein expression that correlated with a decrease in NCC activity/expression. The reasons for the discrepancies between these observations, which have not been replicated (38, 39), and our own, which suggest a selective role for α1D-adrenoceptors, are unclear but may include species differences. Significantly, a role for α1D-adrenoceptor signaling is supported by a study in C57B1/6J mice (38), which confirmed a role of β-adrenoceptors and provided evidence that α1D-adrenoceptors play a synergistic, albeit minor, part in acute NCC regulation. The minor role of α1D-adrenoceptors in mice was assessed as the change in NCC phosphorylation after 30 min of α1D- and/or β-adrenoceptor stimulation (38). In comparison, the present study evaluated NCC activity, mRNA and protein expression, and phosphorylation after 3 wk of NE coinfused with a selective α1D- or β-antagonist during both NS and HS intake.

Although these results suggest that β-adrenoceptors may play a more important role in short-term NCC regulation, our findings highlight that α1D-adrenoceptor signaling is relevant in the long-term regulation of NCC. A long-term regulatory role of α1D-adrenoceptors in NCC activity is supported by our observation that α1D-adrenoceptor antagonism initiated after 3 wk of HS intake in NE-infused rats, a time point at which salt-sensitive hypertension is established, attenuates the salt-sensitive component of hypertension and restores suppression of NCC activity. It should be noted that the acute adrenoceptor stimulation experiments were performed in mice on a NS diet and blood pressure was not measured (38), raising the possibility that a role for α1D-adrenoceptors in NCC regulation also exists in mice but is limited to the context of salt-sensitive hypertension. It is also worth considering the contrasting approaches of isolated adrenoceptor stimulation using pharmacological agonists versus adrenoceptor antagonism during NE infusion, which may better reflect the balance of α/β-adrenoceptor activation occurring during increased sympathetic outflow as seen in salt-sensitive hypertension in humans. We acknowledge that there is a role of β- but not α-adrenergic pathways in mouse DCT cells in modulating NCC activity via a Kir4.1/Kir5.1 pathway that was not assessed in the present study (9).

Further supporting our in vivo observation of a role for α1D-adrenoceptor signaling, α1D-adrenoceptor mRNA expression was increased by NE infusion and remained elevated in NE-infused rats during HS intake. Although the role of the α1D-adrenoceptor in the regulation of NCC is unknown, studies have shown α1D-adrenoceptor localizing to renal tubules and the renal pelvic wall (18, 49). Future studies are required to delineate the role of the α1D-adrenoceptor in our proposed signal transduction pathway. In another study (37), salt-sensitive hypertension was attenuated in α1D-adrenoceptor knockout mice, raising the possibility that increased signaling via α1D-adrenoceptors contributes to salt sensitivity in our model. Moreover, mRNA expression of α2B1- and α2B2-adrenoceptors is suppressed during HS intake in saline-infused rats but not in NE-infused rats. A prior study (19) demonstrated that dietary Na+-evoked suppression of renal α2D-adrenoceptor signaling might contribute to the sympathoinhibitory renorenal reflex that promotes Na+ homeostasis in salt-resistant models. It is therefore possible that the failure to suppress α2B1- and α2B2-adrenoceptor mRNA during HS intake in NE-infused animals is functionally relevant. Although β2-adrenoceptor mRNA ex-
pression is reduced during HS intake in saline-infused rats, a similar reduction is observed in NE-infused rats irrespective of Na⁺ intake, supporting a specific role for α-adrenergic signaling in our model of salt sensitivity.

Interpretation of our findings is somewhat limited by the systemic administration of NE, which can influence blood pressure via activation of adrenoreceptors outside the kidney. Studies beyond the scope of the present work may include assessment of these adrenergic pathways in the Dahl salt-sensitive rat, in which the specific role of renal NE could be further elucidated using bilateral renal denervation. However, our model reasonably reflects the state of global sympathoexcitation observed in salt sensitivity (8, 12) and neurogenic hypertension. Additionally, our findings may be particularly relevant to the context of human aging, which is characterized by lifelong exposure to excessive dietary Na⁺ intake (28), increased sympathetic tone (10), and dramatic increases in the prevalence of hypertension (26) and the salt sensitivity of blood pressure (21). A final limitation of our approach is our direct measurement of blood pressure in acutely instrumented animals. However, this approach provides the ability to measure blood pressure and the physiological activity of NCC in the same animal and represents the only way to assess in vivo NCC activity (4, 40) and reproduces and replicates our prior published data using this experimental approach (43).

Collectively, our findings suggest that NE drives the development of salt-sensitive hypertension in Sprague-Dawley rats, in part via an α1-adrenergic-gated, WNK-SPAK/OsR1 signaling pathway that promotes NCC activity. Furthermore, we have demonstrated that targeting this pathway prevents the development of salt-sensitive hypertension and, critically, abolishes salt sensitivity in established hypertension, which better reflects the stage at which human hypertension is diagnosed and treated. The strong role of WNKs suggested by our findings is especially important, given the recent development of salt-sensitive hypertension and, critically, that WNKs is a putative activator of WNK4 and NCC. Am J Physiol Renal Physiol 315: F734–F745, 2018. doi: 10.1152/ajprenal.00145.2018.

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

AUTHOR CONTRIBUTIONS
R.D.W. conceived and designed research; A.A.F., F.P., K.K., K.R.W., E.S.P., R.S.H., and R.D.W. performed experiments; A.A.F., F.P., K.K., K.R.W., R.S.H., and R.D.W. analyzed data; A.A.F., F.P., K.K., R.R.W., R.S.H., and R.D.W. interpreted results of experiments; A.A.F. and F.P. prepared figures; A.A.F. and F.P. drafted manuscript; A.A.F., F.P., and R.D.W. edited and revised manuscript; A.A.F., F.P., K.K., R.R.W., R.S.H., and R.D.W. approved final version of manuscript.

GRANTS
This work was supported by National Institutes of Health (NIH) Grants R56-AG05767, R01-HL-139867, R01-HL-141406, R01-HL-107330, and K02-HL-112718 and American Heart Association Grants 16MM32090001 and 17GRNT33670023 (to R. D. Wainford), NIH Grant F31-DK-116501 (to A. A. Frame), MERIT Award IOB00322 and NIH Grant R01-HL-139867 (to R. S. Hoover), and funds from Boston University’s Undergraduate Research Opportunities Program (to E. Fauudo) and an American Society of Nephrology Foundation for Kidney Research Pre-Doctoral Fellowship award and American Physiological Society William Townsend Porter Physiology Development Fellowship award (to F. Puleo).

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

AUTHOR CONTRIBUTIONS
R.D.W. conceived and designed research; A.A.F., F.P., K.K., K.R.W., E.S.P., R.S.H., and R.D.W. performed experiments; A.A.F., F.P., K.K., K.R.W., R.S.H., and R.D.W. analyzed data; A.A.F., F.P., K.K., R.R.W., R.S.H., and R.D.W. interpreted results of experiments; A.A.F. and F.P. prepared figures; A.A.F. and F.P. drafted manuscript; A.A.F., F.P., and R.D.W. edited and revised manuscript; A.A.F., F.P., K.K., R.R.W., R.S.H., and R.D.W. approved final version of manuscript.

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