Norepinephrine-evoked salt-sensitive hypertension requires impaired renal sodium chloride cotransporter activity in Sprague-Dawley rats

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Walsh KR, Kuwabara JT, Shim JW, Wainford RD. Norepinephrine-evoked salt-sensitive hypertension requires impaired renal sodium chloride cotransporter activity in Sprague-Dawley rats. Am J Physiol Regul Integr Comp Physiol 310: R115–R124, 2016. First published November 25, 2015; doi:10.1152/ajpregu.00514.2014.—Recent studies have implicated a role of norepinephrine (NE) in the activation of the sodium chloride cotransporter (NCC) to drive the development of salt-sensitive hypertension. However, the interaction between NE and increased salt intake on blood pressure remains to be fully elucidated. This study examined the impact of a continuous NE infusion on sodium homeostasis and blood pressure in conscious Sprague-Dawley rats challenged with a normal (NS; 0.6% NaCl) or high-salt (HS; 8% NaCl) diet for 14 days. Naïve and saline-infused Sprague-Dawley rats remained normotensive when placed on HS and exhibited dietary sodium-evoked suppression of peak natriuresis to hydrochlorothiazide, suggesting impaired NCC activity contributes to the development of salt sensitivity [peak natriuresis to hydrochlorothiazide (μeq/min) Naïve+NS: 9.4 ± 0.2 vs. Naïve+HS: 7 ± 0.1; P < 0.05; NE+NS: 11.1 ± 1.1; NE+HS: 10.8 ± 0.4]. NE infusion did not alter NCC expression in animals maintained on NS; however, dietary sodium-evoked suppression of NCC expression was prevented in animals challenged with NE. Chronic NCC antagonism abolished the salt-sensitive component of NE-mediated hypertension, while chronic ANG II type 1 receptor antagonism significantly attenuated NE-evoked hypertension without restoring NCC function. These data demonstrate that increased levels of NE prevent dietary sodium-evoked suppression of the NCC, via an ANG II-independent mechanism, to stimulate the development of salt-sensitive hypertension.

NCC; salt-sensitive hypertension; norepinephrine; sodium homeostasis; sympathetic nervous system

HYPERTENSION, a significant public health burden, contributes to deaths from stroke, myocardial infarction, and kidney failure, making hypertension the single greatest risk factor for premature mortality. Salt-sensitive hypertension occurs in ~50% of hypertensive patients and results in a three-fold increase in the risk of adverse cardiovascular events (9, 19, 39). It is well established that multiple factors contribute, in an integrated fashion, to the pathophysiology of salt-sensitive hypertension, and current evidence points to the kidney playing a pivotal role in the long-term control of blood pressure through its essential role in regulating sodium homeostasis (2, 7, 13). Recently, there has been increased interest in delineating the interactions between the sympathetic nervous system and the kidney, which function to regulate sodium reabsorption. Increased dietary salt intake in salt-resistant phenotypes results in the suppression of neural, humoral, and renal sodium-retaining mechanisms (5, 16, 24). In contrast, increased activity of the sympathetic nervous system is thought to play a key role in the pathogenesis of salt-sensitive hypertension by triggering an increase in renal sodium and water retention (8, 18, 25).

The epithelial sodium channel (ENaC) is an amiloride-sensitive sodium channel located at the apical membrane of polarized epithelial cells in the collecting tubules in the kidney (29). Mutations in ENaC result in Liddle’s syndrome and have been linked to salt sensitivity in human patient populations (27, 31). The sodium chloride cotransporter (NCC) is a thiazide-sensitive transporter that is predominantly found on the apical side of the distal convoluted tubule (DCT; Ref. 12). The importance of the NCC in blood pressure regulation and sodium homeostasis is showcased in the genetic disorder Gitelman’s syndrome, in which loss-of-function mutations in the NCC result in salt-wasting, hypokalemia, and hypertension (28). In the salt-resistant Sprague-Dawley rat, elevations in dietary salt intake suppress sympathetic outflow and circulating norepinephrine (NE) levels (14, 15), in addition to rapidly and persistently reducing the expression of the NCC (40). The physiological process of downregulating the NCC in response to increased dietary salt intake is hypothesized to facilitate sodium homeostasis and normotension (40). However, investigations into the direct impact of increased NE levels on the expression of the NCC during normal dietary salt intake have produced conflicting evidence. In the Sprague-Dawley rat, increased plasma NE content, achieved via a subcutaneous osmotic minipump infusion of NE, in animals maintained on a normal salt intake evoked hypertension without impacting the renal expression of the NCC (30). In contrast, recent studies conducted in C57BL/6J mice have reported that a chronic subcutaneous NE infusion results in the development of hypertension and the upregulation of both total and phosphorylated NCC expression during normal salt intake (22, 32). However, in these studies, the impact of a high-salt intake in combination with a subcutaneous NE infusion on NCC expression was not investigated. In addition to the recent focus of the actions of NE on the NCC, ANG II, a potent vasconstrictor and sodium-retaining hormone (20), has been reported to alter...
both the activity and phosphorylation of the NCC via actions on the angiotensin type 1 receptor (AT1); Refs. 26, 34, 36.

In this study, we hypothesize that salt-sensitive hypertension is driven, in part, by a failure to downregulate renal NCC activity in the presence of excess circulating NE during high dietary salt intake. The following studies are designed to examine the impact of excess circulating NE and increased dietary salt intake, both individually and in combination, on sodium homeostasis, blood pressure regulation, and NCC function and expression in conscious male Sprague-Dawley rats. Furthermore, chronic pharmacological antagonism of the AT1 receptor examined the potential role of the renin-angiotensin system (RAS) in the development of NE-evoked salt-sensitive hypertension.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN; 275–299 g body wt) were individually housed in a temperature (range 68–79°F)- and humidity (range 30–70%)-controlled environment under a 12:12-h light-dark cycle. Following the completion of surgical procedures, rats were randomly assigned to a standard normal salt (NS) rodent diet (Teklad Global Diet, Harlan Laboratories, Indianapolis, IN; Teklad Global 18% protein rodent diet no. 2918, 18% protein, 5% crude fat, 5% fiber, total NaCl content 0.6% (102 meq Na+/kg)) or a high-salt (HS) rodent diet [Test Diet, St. Louis, MO; basal diet no. 5G01, 22% protein, 5.5% crude fat, 5% fiber, modified to contain total NaCl content 8% (1,378 meq Na+/kg)] and tap water ad libitum for a 14-day experimental period. All protocols were approved by the Boston University School of Medicine Institutional Animal Care and Use Committee, and all procedures were conducted in accordance with the National Institutes of Health’s “Guide for the Care and Use of Laboratory Animals.”

Surgical Procedures

Subcutaneous osmotic minipump implantation. Animals were anesthetized (sodium methohexital, 20 mg/kg ip) and surgically instrumented with an osmotic minipump (Alzet, osmotic pump model 2ML2, Palo Alto, CA) that was placed subcutaneously in the subscapular region. Following subcutaneous osmotic minipump surgical placement, all animals were returned to their home cage following administration of penicillin (300,000 units/ml, 0.3 ml im).

Acute femoral vein, artery, and bladder cannulation. Following 14 days of NS or HS intake, all animals were anesthetized (sodium methohexital, 20 mg/kg ip, supplemented with 10 mg/kg iv, as required). Once anesthetized, rats were instrumented with catheters in the left femoral artery, left femoral vein, and bladder for the measurement of arterial blood pressure, intravenous administration of saline and/or drugs, and renal function, respectively (15, 37, 38). Rats were then placed in a Plexiglas holder, and an intravenous infusion of isotonic saline (20 μl/min) was maintained for a 2-h surgical recovery period prior to experimentation to enable the animal to regain full consciousness and for cardiovascular/renal excretory functions to stabilize (15, 37, 38). Mean arterial pressure (MAP) and heart rate (HR) were continuously recorded via the surgically implanted femoral artery cannula using computer-driven BIOPAC data acquisition software (MP150 and AcqKnowledge 3.8.2; BIOPAC Systems, Goleta, CA) connected to an external pressure transducer (P23XL; Vigo Spectramed, Oxnard, CA; Refs. 15, 37, 38). Note that the zero level of the pressure transducer corresponded to heart level.

Experimental Treatment Groups

Naïve animals. Naïve animals were randomly assigned to receive a NS or HS diet for a 14-day experimental period (n = 6/group).

Isotonic saline vehicle infusion. Animals underwent implantation of an osmotic minipump delivering a subcutaneous infusion of isotonic saline (flow rate 5 μl/h) prior to random assignment to either a NS or HS diet for a 14-day experimental period (n = 6/group).

Norepinephrine infusion. Animals underwent implantation of an osmotic minipump delivering a subcutaneous infusion of NE (Sigma, St. Louis, MO; cat. no. A7256) dissolved in isotonic saline (NE: 600 ng/min, flow rate 5 μl/h; Ref. 30) prior to random assignment to either a NS or HS diet for a 14-day experimental period (n = 6/group).

DMSO/saline vehicle infusion. Animals underwent implantation of an osmotic minipump delivering a subcutaneous infusion of DMSO/isotonic saline (50:50 solution, flow rate 5 μl/h) prior to random assignment to either a NS or HS diet for a 14-day experimental period (n = 6/group).

Hydrochlorothiazide infusion. Animals underwent implantation of an osmotic minipump delivering a subcutaneous infusion of NE (NS: 600 ng/min, flow rate 5 μl/h; Ref. 30), in combination with hydrochlorothiazide (HCTZ; Sigma, St. Louis, MO; cat. no. H4759) dissolved in DMSO/isotonic saline (50:50 solution; HCTZ: 4 mg/kg·1-day·1; flow rate 5 μl/h; Ref. 4) prior to random assignment to either a NS or HS for a 14-day experimental period (n = 6/group).

Losartan infusion. Animals underwent implantation of an osmotic mini-pump delivering a subcutaneous infusion of NE (NS: 600 ng/min, flow rate 5 μl/h; Ref. 30) in combination with losartan (Tokyo Chemical Industry, Tokyo, Japan; cat. no. L2032) dissolved in DMSO/isotonic saline (50:50 solution; losartan: 3 mg/kg/day, flow rate 5 μl/h; Ref. 17) prior to random assignment to either a NS or HS for a 14-day experimental period (n = 6/group).

Acute Experimental Protocols

The following cardiovascular, renal sodium transporter activity, and autonomic function protocols were performed consecutively in a single experiment in each animal following 14 days of NS or HS intake.

Cardiovascular function. Following a 2-h surgical recovery period, baseline MAP was recorded continuously over a 30-min period in conscious rats via the surgically implanted femoral artery cannula (n = 6/group; Refs. 15, 38). In Figs. 1A, 4A, and 6A, the presented value for MAP represents the average obtained over the entire 30-min period in which baseline MAP was recorded.

Renal sodium transporter activity. Following the completion of the cardiovascular function protocol, a renal sodium transporter activity protocol was initiated. All animals received an intravenous infusion of isotonic saline (flow rate 20 μl/min) for 1 h, followed by an intravenous bolus of amiloride (2 mg/kg) preceding a 1-h intravenous infusion of amiloride (2 mg/kg, flow rate 20 μl/min; Ref. 4), and an intravenous bolus of HCTZ (2 mg/kg) preceding a 1-h intravenous infusion of amiloride (2 mg/kg, flow rate 20 μl/min; Ref. 4) in combination with HCTZ (2 mg/kg, flow rate 20 μl/min; Ref. 4). Throughout the 3-h protocol, HR and MAP were continuously monitored, and urine was collected in 10-min intervals to assess peak natriuresis to intravenous amiloride or HCTZ (n = 6/group). The peak natriuretic response (∆UNaV; μeq/min) was determined by subtracting the baseline UNaV value from the maximum natriuretic value observed during each hour of drug infusion (i.e., intravenous amiloride or intravenous amiloride+HCTZ). Baseline UNaV values were determined by averaging the UNaV values from the last two 10-min time points during the previous hours of the study (hour 1: intravenous saline (40–50 min, 50–60 min) or hour 2: intravenous amiloride (100–110 min, 110–120 min)). The maximum natriuretic response to amiloride and amiloride+HCTZ occurred during the 10–20 min and 20–30 min time points post-drug infusion, respectively.

Autonomic function protocol. Following completion of the renal sodium transporter activity protocol, the peak change in MAP in response to an intravenous bolus of hexamethonium (30 mg/kg; Refs.
15 and 38) was assessed. Baseline MAP was determined as the average MAP recorded over a 10-min control period prior to hexa-
methonium injection. After baseline MAP measurement, animals received an intravenous bolus of hexamethonium, and blood pressure was monitored for an additional 30-min period. The peak depressor response, assessed over a 60-s period, occurred within 5 min postin-
jection. Following protocol completion, rats were decapitated while conscious, and both kidneys were collected and immediately frozen at −80°C for measurement of the NCC in the kidney cortex.

Metabolic Balance Studies

Metabolic balance studies were conducted in all treatment groups on day 13 of their respective dietary sodium intake period following a 48-h acclimatization to the metabolic cages. Rats were individually housed in metabolic cages (model 18cv, Fenco, Cataumet, MA), with external food containers and water bottles. Metabolic cages were equipped with a double-fine mesh screen that allowed separation of food and feces from urine that was collected into beakers that contained a layer of mineral oil to prevent evaporation. Rats were given ad libitum access to their respective assigned diet and tap water for 24 h. Measurements were made for food and water consumption and urine output during the 24-h period (15, 38). Daily water balance was determined by calculating the difference between water intake and urine output. Daily sodium balance was determined by calculating the difference between sodium intake (dietary sodium intake) and sodium output (urinary sodium excretion).

Analytical Techniques

Urinary volume was determined gravimetrically, assuming 1 g = 1 ml. Urinary and plasma sodium concentration was measured by flame photometry (model 943; Instrumentation Laboratories, Bedford, MA; Refs. 15, 37, 38). Plasma hematocrit (Hct) was determined using a micro-hematocrit centrifuge (Adams Readacrit, Clay Adams, NJ). Hct was used to calculate estimated plasma volume (EPV) and estimated blood volume (EBV) using the following equations: 

\[ \text{EPV} = \frac{0.065 \times \text{vole of blood (ml)}}{(100 - \text{Hct})} \]

\[ \text{EBV} = \frac{\text{EPV} 	imes 100}{(100 - \text{Hct})} \]

Plasma Renin Activity and Plasma Norepinephrine Measurement

In separate groups of animals, naïve Sprague-Dawley and NE-
infused Sprague-Dawley rats were placed on a NS or HS diet for the 14-day experimental protocol. At the end of the 14-day experimental period, animals were decapitated while conscious and whole blood was collected. Plasma renin activity was determined via ELISA (Immuno-Biological Labs America, Minneapolis, MN; cat. no. IB59131). Plasma NE content was determined via ELISA (Immuno-Biological Labs America, Minneapolis, MN; cat. no. A9044) in 0.1% PBS-Tween for 1 h at room temperature. Bound antibodies were visualized using chemiluminescence (GE signal enhancer; GE, Buckinghamshire, UK). Densitometric analysis was performed using Quantity One software (Bio-Rad, Hercules, CA), and band densities were normalized to β-actin.

Statistical Analysis

Data are expressed as means ± SE. Differences between groups were assessed by a one-way ANOVA, followed by a Tukey post hoc test, to compare variations among the groups. A two-tailed Student’s t-test was used to assess the difference between diet groups for the NCC expression quantification. Statistical analysis was carried out using a software program (GraphPad Prism version 6; GraphPad software, La Jolla, CA). Statistical significance is defined as \( P < 0.05 \).

RESULTS

High Salt Intake Exacerbates Norepinephrine-Induced Hypertension

When naïve or subcutaneous infused saline-treated Sprague- Dawley rats were challenged with a HS diet for 14 days, we did not detect any difference in baseline MAP compared with animals maintained on a NS diet (Fig. 1A). In contrast to a

Fig. 1. Basal mean arterial pressure (MAP, mmHg) (A) and index of salt-
sensitivity (B) were determined from a 24-h sodium excretion and baseline
MAP in naïve conscious male Sprague-Dawley rats and in Sprague-Dawley rats receiving either a subcutaneous isotonic saline infusion or a subcutaneous norepinephrine (NE; 600 ng/min) infusion maintained on either a normal (0.6% NaCl) or a high-salt (8% NaCl) diet for 14 days. Data are expressed as means ± SE; \( n = 6 \) /group. *\( P < 0.05 \) vs. respective 0.6% NaCl diet group value. **\( P < 0.05 \) vs. respective naive group value. ***\( P < 0.05 \) vs. respective saline group value.
subcutaneous saline infusion, a 14-day subcutaneous infusion of NE at a rate of 600 ng/min resulted in the development of hypertension in animals maintained on a NS diet [Fig. 1A; MAP (mmHg) Naïve+NS: 128 ± 2; subcutaneous saline+NS: 125 ± 2 vs. subcutaneous NE+NS: 151 ± 3; P < 0.05]). The magnitude of hypertension evoked by a subcutaneous NE infusion was exacerbated when the animals were maintained on a HS diet [Fig. 1A; MAP (mmHg) subcutaneous NE+NS: 151 ± 3 vs. subcutaneous NE+HS: 172 ± 4; P < 0.05]. Illustrated as an index of the salt sensitivity of blood pressure, both naïve and subcutaneous saline-infused Sprague-Dawley rats exhibit a classical salt-resistant phenotype. The subcutaneous infusion of NE caused a significant reduction in the slope of the chronic pressure-natriuresis relationship in Sprague-Dawley rats (Fig. 1B), reflecting the increased salt sensitivity of blood pressure. Additionally, Sprague-Dawley rats placed on a HS diet or receiving a subcutaneous NE infusion showed no significant differences in 24-h sodium or water balance (Table 1). Furthermore, the combination of a subcutaneous NE infusion and increased salt intake did not significantly alter 24-h sodium or water balance (Table 1). When estimated plasma (EPV) and blood volume (EBV) was calculated in these groups of animals, we observed no significant effect of increased dietary sodium intake or NE infusion, alone or in combination, on EPV or EBV (Table 2).

Table 1. Twenty-four hour water and sodium balance listed for naïve Sprague-Dawley rats and Sprague-Dawley rats receiving a subcutaneous isotonic saline, 50:50 solution of DMSO and isotonic saline, NE, or NE and HCTZ infusion maintained on either a normal (0.6% NaCl) or high-salt (8% NaCl) diet for 14 days

<table>
<thead>
<tr>
<th>Treatment</th>
<th>24-h H2O Balance, ml</th>
<th>24-h Sodium Balance, meq</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.6% NaCl</td>
<td>8% NaCl</td>
</tr>
<tr>
<td>Naïve</td>
<td>19.1 ± 3.2</td>
<td>24.3 ± 4.0</td>
</tr>
<tr>
<td>Saline</td>
<td>26.2 ± 2.6</td>
<td>33.2 ± 4.2</td>
</tr>
<tr>
<td>DMSO Saline</td>
<td>21.5 ± 4.2</td>
<td>34.1 ± 6.0</td>
</tr>
<tr>
<td>NE, 600 ng/min</td>
<td>13.7 ± 2.6</td>
<td>19.5 ± 2.5</td>
</tr>
<tr>
<td>NE, 600 ng/min, and HCTZ, 4 mg/kg⁻¹·day⁻¹</td>
<td>16.3 ± 4.3</td>
<td>13.8 ± 2.3</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE (n = 6/group). NE, norepinephrine; HCTZ, hydrochlorothiazide.

Table 2. EPV and EBV in milliliters listed for naïve Sprague-Dawley rats and Sprague-Dawley rats receiving a subcutaneous infusion of isotonic saline or 600 ng/min NE, maintained on either a normal or a high-salt diet for 14 days

<table>
<thead>
<tr>
<th>Treatment</th>
<th>EPV, ml</th>
<th>EBV, ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naïve 0.6% NaCl</td>
<td>12.2 ± 0.4</td>
<td>21.0 ± 0.2</td>
</tr>
<tr>
<td>Naïve 8% NaCl</td>
<td>12.5 ± 0.2</td>
<td>21.7 ± 0.2</td>
</tr>
<tr>
<td>Saline 0.6% NaCl</td>
<td>11.9 ± 0.2</td>
<td>22.0 ± 0.4</td>
</tr>
<tr>
<td>Saline 8% NaCl</td>
<td>11.8 ± 0.3</td>
<td>21.1 ± 0.2</td>
</tr>
<tr>
<td>NE 0.6% NaCl</td>
<td>12.2 ± 0.4</td>
<td>21.8 ± 0.7</td>
</tr>
<tr>
<td>NE 8% NaCl</td>
<td>11.9 ± 0.2</td>
<td>21.6 ± 0.2</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE (n = 5 or 6/group). EPV, estimated plasma volume; EBV, estimated blood volume.

Norepinephrine Infusion Prevents Dietary Sodium-Evoked Suppression of NCC Activity

To assess the impact of NE infusion on vascular tone, we challenged animals with the ganglionic blocker hexamethonium via an acute intravenous bolus injection. As illustrated (Fig. 2A), increased dietary sodium intake did not alter the peak depressor response following ganglionic blockade in naïve and subcutaneous saline-infused Sprague-Dawley rats. However, NE infusion significantly increased the peak depressor response to hexamethonium, a response that was not altered following 14 days of HS intake (Fig. 2A). Next, we assessed the impact of NE infusion on the activity of two key renal sodium transporters, ENaC and NCC. The peak natriuretic response to amiloride in naïve and saline-treated groups was suppressed on a HS diet [Fig. 2B; peak ΔUNaV to intravenous amiloride (μeq/min) Naïve+NS: 7.7 ± 0.5 vs. Naïve+HS: 4.2 ± 1; P < 0.05]. NE infusion did not impact the dietary sodium-evoked suppression of the peak natriuretic response to amiloride (Fig. 2B; peak ΔUNaV to intravenous amiloride (μeq/min): subcutaneous NE+NS: 9.4 ± 1 vs. subcutaneous NE+HS: 3.7 ± 1.6; P < 0.05). Both naïve and subcutaneous saline-infused rats exhibited dietary sodium-evoked suppres-
showed dietary sodium-evoked suppression of renal NCC expression (Fig. 3, A and B), mirroring our physiology results. Chronic NE infusion did not significantly change NCC expression in animals maintained on a NS intake. In contrast to the result obtained in saline-infused rats, animals receiving a NE infusion and maintained on HS failed to show a dietary sodium-evoked decrease in NCC expression (Fig. 3, A and B).

**Chronic NCC Antagonism Abolishes the Salt-Sensitive Component of Norepinephrine-Evoked Hypertension**

Following our observation that a NE infusion prevented the sodium-evoked suppression of NCC function and expression (Figs. 2C, 3, A and B), we elected to chronically antagonize the NCC with HCTZ during subcutaneous NE infusion. A subcutaneous DMSO/saline vehicle infusion did not alter the salt-resistant phenotype of the Sprague-Dawley rat (Fig. 4, A and B) or the sodium-evoked suppression of the peak natriuresis to HCTZ (Fig. 5B). The coinfusion of HCTZ with NE did not prevent the development of hypertension in animals maintained on a NS diet. In these animals, the magnitude of hypertension was comparable to that observed in NE-infused animals [Fig. 4A; MAP (mmHg) subcutaneous NE+NaCl: 151 ± 3; subcutaneous NE+HCTZ+NaCl: 145 ± 1]. Significantly, the coinfusion of NE with HCTZ during a HS intake abolished the salt-sensitive component of NE-evoked hypertension, reducing the MAP to a level similar to that observed in NE-treated animals maintained on NS (Fig. 4A). When plotted as an index of the salt sensitivity of blood pressure, coinfusion of NE and HCTZ significantly increased the slope of the chronic pressure-natriuresis relationship vs. animals receiving a subcutaneous infusion of NE alone (Fig. 4B), indicating a reduction in the salt sensitivity of blood pressure. The NE+HCTZ coinfusion did not prevent dietary sodium-evoked suppression of the peak natriuretic response to amiloride (Fig. 5A). Demonstrating the efficacy of our chronic antagonism of the NCC, an acute intravenous infusion of HCTZ did not evoke natriuresis in animals receiving a subcutaneous NE+HCTZ coinfusion (Fig. 5B). Additionally, DMSO/saline vehicle or coinfusion of NE+HCTZ did not significantly alter 24-h sodium or water balance regardless of salt intake (Table 1). When EPV and EBV were calculated in these groups of animals, we observed no significant effect of increased dietary sodium intake or drug infusion on EPV or EBV (Table 4).

**Norepinephrine Infusion Prevents Dietary Sodium-Evoked Suppression of NCC Expression**

Following the completion of all acute experiments, kidney cortex tissue was harvested for analysis of NCC expression from the same saline- and NE-infused rats for which physiological data are presented in Figs. 1 and 2. When challenged with HS for 14 days, normotensive saline-treated animals showed dietary sodium-evoked suppression of renal NCC expression (Fig. 3, A and B), mirroring our physiology results. Chronic NE infusion did not significantly change NCC expression in animals maintained on a NS intake. In contrast to the result obtained in saline-infused rats, animals receiving a NE infusion and maintained on HS failed to show a dietary sodium-evoked decrease in NCC expression (Fig. 3, A and B).

**Table 3. Estimated plasma renin activity expressed as ANG I generation and plasma NE content listed for naïve Sprague-Dawley rats and Sprague-Dawley rats receiving a subcutaneous infusion of 600 ng/min NE maintained on a normal or high-salt diet for 14 days**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PRA Expressed as ANG I Generation, ng·ml⁻¹·h⁻¹</th>
<th>Plasma NE, nmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naïve 0.6% NaCl</td>
<td>5.1 ± 0.3</td>
<td>61.6 ± 4.5</td>
</tr>
<tr>
<td>Naïve 8% NaCl</td>
<td>1.8 ± 0.3</td>
<td>36.7 ± 4.2*</td>
</tr>
<tr>
<td>NE 0.6% NaCl</td>
<td>1.3 ± 0.2</td>
<td>104.3 ± 8.2*</td>
</tr>
<tr>
<td>NE 8% NaCl</td>
<td>0.8 ± 0.1</td>
<td>118.4 ± 7.3*</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE (n = 5/group). PRA, plasma renin activity. *P < 0.05 vs. naïve 0.6% NaCl group value. **P < 0.05 vs. naïve 8% NaCl group value.

**Fig. 3. A:** representative immunoblots illustrating sodium chloride cotransporter (NCC) expression in kidney cortex tissue. B: quantification of renal NCC expression in male Sprague-Dawley rats receiving either a subcutaneous isotonic saline or NE (600 ng/min sc) infusion maintained for 14 days on a normal (0.6% NaCl) or high-salt (8% NaCl) diet. Data are expressed as means ± SE (n = 6/group). *P < 0.05 vs. respective 0.6% NaCl group value.

**Fig. 4. A:** saline or NE (600 ng/min sc) infusion maintained for 14 days on a normal (0.6% NaCl) or high-salt (8% NaCl) diet. Data are expressed as means ± SE (n = 6/group). *P < 0.05 vs. respective 0.6% NaCl group value.
Chronic AT1 Receptor Antagonism Prevents Norepinephrine-Evoked Hypertension, but Does Not Restore Sodium-Evoked NCC Impairment

To determine the contribution of the RAS to impaired NCC function during NE infusion and HS intake, we co-infused Sprague-Dawley rats with NE and losartan, an AT1 receptor antagonist. A subcutaneous infusion of NE+losartan significantly reduced baseline MAP for animals on a NS diet compared with NE infusion alone [Fig. 6A; MAP (mmHg) subcutaneous NE+NS: 151 ± 3 vs. subcutaneous NE+losartan+NS: 135 ± 3; P < 0.05]. A NE+losartan coinfusion also prevented the development of salt sensitivity [Fig. 6A; MAP (mmHg) subcutaneous NE+HS: 172 ± 4 vs. subcutaneous NE+losartan+HS: 138 ± 3; P < 0.05]. Dietary sodium-evoked suppression of peak natriuresis to amiloride was observed in animals receiving a subcutaneous infusion of NE+losartan [Fig. 6B; peak ΔUNaV to intravenous amiloride (μeq/min) subcutaneous NE+losartan+NS: 7.8 ± 1 vs. subcutaneous NE+losartan+HS: 4.15 ± 1; P < 0.05]. However, dietary sodium evoked suppression of natriuresis to HCTZ was not observed following NE+losartan coinfusion [Fig. 6C; peak ΔUNaV to intravenous HCTZ (μeq/min) subcutaneous NE+losartan+NS: 11.5 ± 2; subcutaneous NE+losartan+HS: 11.3 ± 2].
an NS diet. Significantly, dietary sodium-evoked suppression of NCC expression was abolished in animals challenged with NE and an elevated salt intake for 14 days, a response correlating with impaired NCC activity and salt sensitivity. Confirming a direct role of the NCC in the development of NE-evoked salt-sensitive hypertension, chronic NCC antagonism with HCTZ abolished the salt-sensitive component of NE-mediated hypertension. Chronic antagonism of AT_1 receptors with losartan was unable to restore NCC function; however, AT_1 receptor blockade was able to significantly reduce NE-evoked increases in MAP in animals maintained on either a NS or HS diet. Collectively, these data show a critical interaction between dietary salt intake and circulating levels of NE that prevent the downregulation of NCC activity and expression to evoke the development of salt-sensitive hypertension in the Sprague-Dawley rat.

Our results demonstrating NE-mediated hypertension are substantiated by the findings of Sonalker et al. (30), in which the same chronic infusion of NE (600 ng/min) resulted in the development of hypertension in Sprague-Dawley rats maintained on a NS diet. Extending the studies of Sonalker et al. (30), we provide evidence that increased plasma NE content—driven by the constant subcutaneous osmotic mini-pump infusion of NE—to levels comparable to those observed in the hypertensive Dahl salt-sensitive rat (38), results in the development of salt sensitivity in the classically salt-resistant Sprague-Dawley rat (14, 15). The development of salt sensitivity during chronic NE infusion is evidenced by a significant increase in MAP during HS intake and a rightward shift of the chronic pressure-natriuresis relationship, reflecting the long-term resetting of the chronic pressure-natriuresis relationship to a new higher set-point blood pressure. Notably, EPV and EBV were not impacted by 14 days of dietary sodium intake or NE infusion in these studies. These data suggest that the observed increases in MAP after the 14-day protocol are not dependent on a long-term increase in blood or plasma volume. However, these studies do not rule out the possibility that transient increases in EPV and/or EBV play a role in the induction and/or development of NE-evoked hypertension. To further investigate the mechanisms contributing to elevated MAP in these NE-infused animals, we assessed the impact of hexamethonium-mediated ganglionic blockade on the peak depressor response. In these studies, we observed no difference in the peak depressor response following ganglionic blockade between NE-infused animals maintained on either a NS or HS diet. In NE-infused animals, we observed a significantly greater drop in blood pressure vs. that observed in saline infused rats, indicating that increased vasoconstriction contributes to the development of NE-evoked hypertension. As observed in control (naïve and saline infused) animals, a HS intake did not significantly affect the depressor response to ganglionic blockade in NE-infused rats, suggesting that there is no impact of sodium intake to increase sympathetic outflow in NE-infused animals. As such, we believe the difference in basal blood pressure observed following ganglionic blockade between NE-infused animals on NS vs. HS is reflective of the baseline blood pressure differences observed prior to hexamethonium administration vs. intrinsic differences in sympathetic activity. The mechanism underlying our observation of an enhanced depressor response after ganglionic blockade in NE-infused animals remains to be determined and highlights the complexity of interpreting the results of ganglionic blockade in the setting of chronic hypertension. In the current experimental paradigm, it would be anticipated that NE infusion would not activate the sympathetic nervous system and may evoke reduced sympathetic outflow. Therefore, we speculate that vascular hypertrophy, as has been reported following ANG II-evoked persistent increases in blood pressure (21), may underlie the enhanced response to ganglionic blockade observed in these studies.

Owing to reports that the Dahl salt-sensitive rat, which exhibits salt-sensitive hypertension and increased plasma NE content (38), exhibits a failure to downregulate certain ENaC subunits during high salt intake (1, 39), we elected to examine the activity of the ENaC in hypertensive NE-infused Sprague-Dawley rats. Our physiological data, generated in conscious animals, indicate that the combination of a NE infusion and a HS intake does not prevent sodium-evoked downregulation of the activity of the ENaC. These data suggest that in the Sprague-Dawley rat, failure to suppress ENaC activity (assessed via peak natriuresis to amiloride) is not involved in the development of NE-evoked salt-sensitive hypertension, a find-
ing in contrast to the ENaC dysregulation observed in the Dahl salt-sensitive rat phenotype (1, 3). The differences between the previously reported impact of salt intake on ENaC in the Dahl salt-sensitive rat and our current report of a lack of effect during chronic NE infusion in the Sprague-Dawley rat may be attributed to 1) the genetic differences between the Dahl salt-sensitive and Sprague-Dawley rat and 2) the multifactorial nature of Dahl salt-sensitive hypertension featuring aberrations in central, vascular, cardiac, hormonal, and renal mechanisms. We elected not to determine renal ENaC subunit expression or to examine the impact of chronic ENaC inhibition on the development of NE-evoked salt sensitivity of blood pressure in the Sprague-Dawley rat for the following reasons: 1) we observed no significant physiological differences in our NE infusion model compared with control animals in terms of the response of ENaC function during HS intake and 2) a prior report stating that chronic ENaC inhibition did not prevent the development of NE-evoked salt-sensitive hypertension in mice (22).

Therefore, our next series of studies focused on the role of the NCC in the development of the salt sensitivity of blood pressure in the Sprague-Dawley rat. Our initial data demonstrate that on a NS intake, the infusion of NE, which evokes hypertension, does not alter the activity (assessed as peak natriuresis to HCTZ) or expression (assessed via immunoblotting) of the NCC. These data support the prior findings that chronic NE exposure does not alter NCC expression in the Sprague-Dawley rat (30) or in mice (33). These data are in direct contrast to data generated in mice reporting that total expression and phosphorylation of NCC are increased by both chronic (22) and acute increases in NE (32). We believe the discrepancy between our data and the data of other laboratories concerning the impact of NE on the expression of the NCC may reflect, in part, a potential species difference in the mechanistic effects of NE on NCC expression. In support of previous studies demonstrating that a HS intake evokes down-regulation of the NCC (40), we observed the downregulation of NCC activity (assessed as peak natriuresis to HCTZ) and protein expression (assessed via immunoblotting) in naïve Sprague-Dawley rats challenged with a HS diet. In contrast, animals that received a NE infusion and a HS diet failed to downregulate the activity of the NCC during HS intake, suggesting a role of impaired NCC function in the pathophysiology of sympathetically mediated salt-sensitive hypertension. These data suggest, that in the Sprague-Dawley rat, contrary to prior observations in mice, the NCC evokes the development of salt-sensitive hypertension through a failure to be downregulated in response to an elevated salt intake vs. a direct action of NE to increase activity and expression of the NCC. We acknowledge that these studies cannot exclude a potential direct effect of increased blood pressure, occurring independently of an interaction between salt and NE, that is influencing the activity of the NCC. To confirm a direct role of the failure to downregulate the activity of the NCC in response to HS in the development of salt sensitivity, we conducted additional studies in which the NCC was chronically antagonized by coinfusing Sprague-Dawley rats with NE + HCTZ for 14 days. The chronic antagonism of the NCC abolished the salt-sensitive component of NE-mediated hypertension and significantly attenuated the reduction in the slope of the chronic pressure-natriuresis relationship. These data provide strong evidence that the NCC plays a key role in the sympathetically mediated regulation of the kidney to drive the development of salt-sensitive hypertension.

To determine the potential contribution of the RAS in this animal model of NE-mediated salt-sensitive hypertension, we coin infused Sprague-Dawley rats with NE + losartan, an AT1 receptor antagonist. The coinfusion of NE + losartan significantly decreased MAP in animals maintained on both NS and HS diets vs. animals receiving a NE infusion. Our data, indicating the suppression of PRA activity during NE infusion, raise the paradoxical issue of how AT1 antagonism reduces blood pressure in the setting of low renin/ANG II levels. These data are in line with prior studies that have demonstrated the efficacy of AT1 antagonism in multiple models of “low renin” hypertension, and we believe that further studies, beyond the scope of the current manuscript, are required to determine the mechanism underlying the apparent counterintuitive abolishment of hypertension during NE infusion by AT1 antagonism. However, when Sprague-Dawley rats receiving a NE + losartan coinfusion were acutely challenged with HCTZ, dietary sodium-evoked suppression of NCC activity was not observed. These data indicate that the failure of the NCC to be downregulated in response to increased dietary salt intake during NE infusion occurs independently of the actions of the RAS. Previous studies have reported that ANG II has direct effects on the abundance, activity, and regulation of the NCC (10, 35, 36). However, a recent study using ANG II receptor type 1a (AT1a) knockout mice reported that NE-induced effects on NCC expression and phosphorylation are not AT1a dependent (32). These data support our results, which also indicate that the effects of NE on the NCC are independent of ANG II. Further, the ability of losartan to prevent NE-evoked hypertension, but not restore HS stimulated downregulation of NCC activity, suggests that NCC activity is regulated by NE via a mechanism that is independent of increased blood pressure. Although low-dose ANG II infusion has been shown (23) to result in hyponatremia in Sprague-Dawley rats, in the current studies, plasma Na⁺ levels in NE-infused rats remained within the normal range (135–145 mmol/l), suggesting that in our NE infusion model, we are not evoking systemic increases in ANG II, at least not to a level that evokes hyponatremia, a hypothesis supported by the NE-evoked suppression of PRA. Our data on the suppression of PRA activity during NE infusion and HS intake substantiate a lack of activation of the RAS in our experimental paradigm. The actions of NE to impact the NCC have been suggested to be dependent on the actions of the β-adrenergic receptor (βAR) (22, 32). Mu et al. (22) reported that stimulation of the β2AR increased NCC activity and sodium retention during the development of salt-sensitive hypertension in mice. However, a recent study has suggested that the β1AR is primarily responsible for mediating the effects of NE on the NCC due to the fact that β1ARs are highly enriched in the DCT (32). Currently, the β-adrenergic receptor subtype responsible for mediating the effects of NE on the NCC remains to be definitively established, as does the impact of dietary salt intake and NE on the renal expression of β-adrenergic receptors.

In conclusion, our results highlight the importance of the interaction between NE and salt, and not just NE alone, to influence the function and expression of the NCC during the pathophysiology of salt-sensitive hypertension. Although we
believe our results are based on an action of NE directly on NCC regulation, we cannot exclude a direct effect of blood pressure preventing the downregulation of the NCC independently of an interaction between salt and NE. In the current studies, NE is being increased systemically, not just locally at the level of the kidney; as such, we are unable to rule out extrarenal effects and acknowledge that circulating NE may not penetrate the kidney to the same extent as local release of NE from sympathetic nerve terminals. However, given that in salt-sensitive human and rat (Dahl salt-sensitive) subjects, there is global sympathetic excitation (6, 11) and that our model of subcutaneous NE infusion is directly comparable with prior studies published in rats (30) and mice (22, 33), we believe we have developed a suitable model to investigate the actions of both salt and NE on sodium homeostasis and blood pressure regulation. Despite the clinical use and well-defined transporter-specific diuretic actions of both HCTZ and amiloride, we recognize that this series of studies relies on their selectivity and that off-target effects must be considered when interpreting the results. Owing to the short-term infusion of amiloride and HCTZ during the acute experimental protocol, we speculate the off-target actions of these drugs are likely limited. In regard to the 14-day infusion of HCTZ, we observed no overt signs of potential hypokalemia that could have impacted our studies. In future studies, beyond the scope of the current investigations, we plan to address the effects of NE and salt on NCC function and the expression in additional rat models of salt-sensitive hypertension (e.g., Dahl salt-sensitive rat vs. Dahl salt-resistant rat). Future studies will also be conducted to investigate the impact of salt and NE on the phosphorylation of NCC via SPAK (SPS1-related proline/alanine-rich kinase) and OxsR1 (oxidative stress response-1) in our animal model to allow for further clarification of the mechanism(s) contributing to the observed dysregulation of NCC activity in Sprague-Dawley rats during NE infusion and high dietary sodium intake.

Perspectives and Significance

The current data highlight the critical interaction between dietary salt intake and increased levels of NE on the activity of the NCC. This work extends our understanding of interactions between the sympathetic nervous system and the kidney in the long-term regulation of sodium homeostasis and blood pressure. These findings challenge recent reports generated in mice of a direct effect of NE to increase expression of the NCC and substantiate a species difference in the response of NCC protein levels to NE infusion. We demonstrate that there is no effect of NE infusion alone on the physiological activity of the NCC in vivo in conscious Sprague-Dawley rats. Our data suggest a crucial impact of NE in preventing the sodium-evoked suppression of NCC activity and expression and support a key role for the NCC in the pathophysiology of salt-sensitive hypertension via actions of the sympathetic nervous system on the kidney. These findings suggest that there are significant species differences in the response to NE and highlight the importance of using multiple animal models to investigate the origins and mechanisms underlying salt sensitivity. We speculate that in hypertensive patients with increased activity of the sympathetic nervous system, there may be an increased risk of developing salt sensitivity vs. patients in which sympathetic activity remains unchanged, possibly using elevated plasma NE levels as a marker of future salt sensitivity.

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DISCLOSURES

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

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