ORIGINAL RESEARCH

Nephron-Specific Disruption of Nitric Oxide Synthase 3 Causes Hypertension and Impaired Salt Excretion

Yang Gao, PhD; Deborah Stuart, BA; Takamune Takahishi, MD, PhD; Donald E. Kohan, MD, PhD

***Background*—**In vitro studies suggest that nephron nitric oxide synthase 3 (NOS3) modulates tubule Na^+ transport.

***Methods and Results*—**To assess nephron NOS3 relevance in vivo, knockout (KO) mice with doxycycline-inducible nephron-wide deletion of NOS3 were generated. During 1 week of salt loading, KO mice, as compared with controls, had higher arterial pressure and Na^+ retention, a tendency towards reduced plasma renin concentration, and unchanged glomerular filtration rate. Chronic high salt-treated KO mice had modestly decreased total NCC and total SPAK/OSR1 versus controls, however percent phosphorylation of NCC (at T^{53}) and of SPAK/OSR1 was increased. In contrast, total and phosphorylated NKCC2 (at $T^{96/101}$) were suppressed by 50% each in KO versus control mice after chronic salt intake. In response to an acute salt load, KO mice had delayed urinary Na^+ excretion versus controls; this delay was completely abolished by furosemide, partially reduced by hydrochlorothiazide, but not affected by amiloride. After 4 hours of an acute salt load, phosphorylated and total NCC were elevated in KO versus control mice. Acute salt loading did not alter total NKCC2 or SPAK/OSR1 in KO versus control mice but increased the percent phosphorylation of NKCC2 (at $T^{96/101}$ and S^{126}) and SPAK/OSR1 in KO versus control mice.

***Conclusions*—**These findings indicate that nephron NOS3 is involved in blood pressure regulation and urinary Na^+ excretion during high salt intake. Nephron NOS3 appears to regulate NKCC2 and NCC primarily during acute salt loading. These effects of NOS3 may involve SPAK/OSR1 as well as other pathways. **(*J Am Heart Assoc*. 2018;7:e009236. DOI: 10.1161/JAHA.118.009236.)**

Key Words: kidney • nephron • nitric oxide synthase • sodium • transporter

Nitric oxide (NO) inhibits NaCl reabsorption in several nephron segments. In isolated thick ascending limb (TAL), NO inhibits Na^+-K^+-$2Cl^-$ cotransporter (NKCC2) activity.[1,2] Studies in isolated collecting duct (CD) and in CD-specific NOS1 knockout (KO) mice suggest that NO inhibits epithelial Na^+ channel (ENaC) activity.[3–5] Nitric oxide may also inhibit proximal tubule Na^+-H^+ exchanger (NHE) activity, although studies in global NO synthase-1 (NOS1) KO mice suggest NO can stimulate proximal tubule fluid absorption.[6] Finally, there are no articles to our knowledge on NO regulation of distal tubule Na^+ transport.

All 3 NOS isoforms (NOS1, NOS2 and NOS3) are expressed within the nephron, although the level of expression of each isoform differs from one another and between cell types. Few studies have examined the nephron-specific role of NOS isoforms in vivo. To our knowledge, no studies have specifically targeted NOS2 within the nephron; while this isoform may prove to play a role within the nephron in blood pressure (BP) control, its low baseline activity and marked induction under pathologic states have made it empirically a less attractive target for examining the role of NOS isoforms in physiological states. Knockout of NOS1 specifically in the macula densa[7] increased the hypertensive response to a high-salt diet, while CD-specific NOS1 knockout (KO) mice had salt-sensitive hypertension and an impaired natriuretic response.[3] In contrast, CD-specific deletion of NOS3 did not affect urinary salt excretion and blood pressure in response to high salt intake.[8] However, other studies, albeit not involving renal cell-specific gene targeting, have suggested that nephron NOS3 may be an important modulator of renal salt handling and BP. L-arginine-induced inhibition of transepithelial Cl^- flux was abolished in TAL from global NOS3 KO mice, but not from global NOS1 or NOS2 KO mice.[9] Further, NOS3 gene transfer into TAL of NOS3 KO mice restored L-arginine-induced inhibition of NaCl reabsorption.[10] Endothelin-1, which inhibits TAL NaCl transport via NO, failed to induce NO production in TAL from NOS3 KO mice.[11] Finally, chronic angiotensin-II-induced hypertension is associated

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Clinical Perspective

What Is New?

- Nephron NOS3 regulates tubule Na^+ transport and blood pressure.
- Nephron NOS3 may regulate NCC and NKCC2, particularly in the setting of an acute salt load.
- Nephron NOS3 may exert its regulatory actions on NCC and NKCC2, at least in part, through SPAR/OSR1.

What Are the Clinical Implications?

- Alterations in nephron NOS3 may contribute to hypertension, including hypertension possibly on a genetic basis, related to high angiotensin-II and in the setting of diabetes mellitus.

with blunted NO production and decreased NOS3 protein expression.[12] Hence, based on the above considerations, the current study was undertaken to determine if nephron NOS3 is, in fact, involved in the physiological regulation of renal salt excretion and BP.

Materials and Methods

Data

The data, analytic methods, and study materials will be/have been made available to other researchers for purposes of reproducing the results or replicating the procedure. Transgenic animals are available through Jackson Laboratory (Bar Harbor, ME) while the floxed NOS3 mouse was provided by Dr Takamune Takahishi at Vanderbilt University.

Animal Care

All animal studies were conducted with the approval of the University of Utah Animal Care and Use Committee in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Generation of Inducible Nephron-Specific NOS3 KO Mice

Floxed NOS3 KO mice were generated as previously described.[13] A targeting construct was made wherein exons 9 to 12 of the *NOS3* gene were flanked by 2 loxP sites; recombination deletes the calmodulin biding site resulting in a non-functional NOS3 protein. To generate inducible nephron-specific NOS3 KO mice, floxed NOS3 mice were bred with mice containing the Pax8-reverse tetracycline transactivator (rtTA) (Pax8 promoter-rtTA confers nephron-specific targeting) and LC-1 transgenes (LC-1 transgene contains doxycyline/rtTA-inducible Cre recombinase and luciferase).[14,15] Mice used in the studies were homozygous for the loxP-flanked NOS3 gene and hemizygous for Pax8-rtTA and LC-1 transgenes. Doxycycline (2 mg/mL) was given in 2% sucrose drinking water to 1-month-old mice for 12 days. Littermates of the same genotype and sex, but without doxycycline treatment were used as controls. Control and NOS3 KO mice aged 3 to 4 months (1:1 male:female) were studied. All mice had a C57BL6 background.

Genotyping and Determination of NOS3 Gene Recombination

Genotyping PCR was performed using tail DNA using: (1) the NOS3 forward primer 5′-GGAGCTTGTGAAGGATAG-3′ and reverse primer 5′-CTGGGTCAAGTTGAAGAG-3′ which yields a 546 bp product from the floxed NOS3 gene and a 440 bp product from the wild-type allele; (2) Pax8-rtTA forward primer 5′-CCATGTCTAGACTGGACAAGA-3′ and reverse 5′-CATCAATG-TATCTTATCATGTCTGG-3′ which yields a 600 bp product; and (3) LC-1 forward primer 5′-TCGCTGCATTACCGGTCGATGC-3′ and reverse primer 5′-CCATGAGTGAACGAACCTGGTCG-3′ which yields a 480 bp product.

To determine NOS3 gene recombination, DNA was isolated from a variety of organs from NOS3 KO mice. NOS3 gene recombination PCR were performed at 94°C for 30 seconds, 65°C for 30 seconds and 72°C for 2 minutes for 35 cycles, using forward primer 5′-GGAGCTTGTGAAGGATAG-3′ and reverse primer 5′-CGCTGAATCCTTGTAGACTG-3′ which yields a 2231 bp non-recombined, and a 475 bp recombined, DNA product.

Sodium Balance Studies

Chronic salt loading

Mice were fed a normal Na^+ diet (0.3% Na^+, Micro-stabilized Rodent Liquid Diet LD101, TestDiet, St. Louis, MO) for 7 days, followed by a low Na^+ diet (0.03% Na^+, TestDiet) for 7 days, and then followed by a high Na^+ diet (3.2% Na^+, TestDiet LD101 with added NaCl) for 7 days. On day 7 of each diet, blood was collected for determination of plasma renin concentration (PRC) and plasma electrolytes. Daily urine samples were collected using metabolic cages, centrifuged at 1500 g for 15 minutes, and supernatants stored at −80°C until assay. Urinary and plasma Na^+ were determined using the EasyVet Analyzer (Medica, Bedford, MA), and net Na^+ balance was calculated as oral Na^+ intake—urinary Na^+ output. Urinary nitrite and nitrate (NOx) concentration was analyzed using a colorimetric assay kit (Cayman Chemical,

Ann Arbor, MI). PRC was measured using an angiotensin-I enzyme immunoassay kit (Peninsula, San Carlos, CA).

Acute salt loading

Control and NOS3 KO mice were given 1.5 mL saline with or without furosemide (10 mg/kg, Sigma), hydrochlorothiazide (15 mg/kg, Sigma) or amiloride (5 mg/kg, Calbiochem, La Jolla, CA) intraperitoneally after fasting overnight, and then placed in metabolic cages without food and water. Urine was collected under oil hourly for 6 to 7 hours. Urinary Na^+ was determined as above. Plasma was collected at the end of hour 7 for determination of K^+ and Na^+.

Blood Pressure Determination

Control and NOS3 KO mice were anesthetized with 2% isoflurane, implanted with radio transmitters with the catheter in the carotid artery, and allowed 5 days recovery. Mice were fed normal, then low and then high Na^+ diets for 7 days each. Blood pressure (BP) was recorded by telemetry (TA11-PAC10, Data Sciences International, St. Paul, MN). Mice were not disturbed during the BP recording period. Blood pressure readings were taken every 10 minutes throughout the day and the average of the entire day's values was used for each daily data point.

Glomerular Filtration Rate Measurement

Glomerular filtration rate (GFR) was measured in control and NOS3 KO mice at baseline condition and after 7 days of high salt intake. Mice were injected with fluorescein isothiocyanate-sinistrin (Mannheim Pharma and Diagnostics, Mannheim, Germany) retro-orbitally (7.5 mg/100 g body weight). The NIC-Kidney (Mannheim Pharma and Diagnostics, Mannheim, Germany) was used to detect fluorescence in the skin on the shaved back over 1 hour. GFR was calculated based on the kinetics of fluorescence decay.

Western Analysis

Whole kidneys from control and NOS3 KO mice were homogenized, and protein isolation and immunoblotting performed as previously described.[8] Antibodies were as follows: NOS3 (1:1000, BD Transduction Labs, Franklin Lakes, NJ), NOS1 (1:500, Santa Cruz, Dallas, TX), NHE3 (1:1000, Millipore, Bedford, MA), phospho (p)-NHE3 (Santa Cruz), ENaC-α, -β, -γ (1:1000, StressMarq, Victoria, BC), p-NCC (T^{53}) and NCC (1:1000, gift from Dr David Ellison, Oregon Health Sciences University), p-NKCC2 ($T^{96/101}$), p-NKCC2 (S^{126}) and NKCC2 (1:1000/1:20 000, gift from Dr Pablo Ortiz, Henry Ford Hospital), p-SPAK (S^{373})/p-OSR1 (S^{325}) (1:1000, Millipore, Temecula, CA), total SPAK and OSR1 (1:1000, gift from Dr Eric Delpire, Vanderbilt University) and mouse monoclonal β-actin (1:1000, Life Technologies, Carlsbad, CA). Secondary horseradish peroxidase-conjugated antibodies were goat anti-mouse immunoglobulin G (1:2000, Santa Cruz) or goat anti-rabbit immunoglobulin G (1:2000, Santa Cruz). Horseradish peroxidase was visualized using the Advance ECL System (GE Healthcare, Piscataway, NJ) or Supersignal West Femto Maximum Sensitivity Substrate (Fisher Scientific, Waltham, MA). Images were obtained and quantified by ImageLab (Bio-Rad, Hercules, CA). All antibodies were initially tested for linearity by loading 1, 2.5, 5, and 10 μg of protein; linear results were obtained for all antibodies between 1 and 5 μg, so 2.5 μg of protein was loaded into each lane for all experiments. Normalizing to ß-actin was still performed, however analysis of the results without ß-actin did not change any significant differences between genotypes.

Statistical Analysis

Specific experiment sample sizes are indicated in the figure legends. The Student *t* test was used to compare differences in continuous parameters on the same day between genotypes (protein expression). Two-way analysis of variance was used to compared differences between control and NOS3 KO mice in urine NOx excretion, PRC, GFR, and plasma electrolytes using genotype and treatment as model terms. Pulse, BP and Na^+ excretion (latter during acute and chronic salt loading) were assessed by 2-way repeated-measures ANOVA with Scheffe post hoc test using genotype and treatment as model terms. An interaction term was included in these models. All analysis was performed using GraphPad Prism 7 software. The criterion for significance was $P<0.05$.

Results

Verification of Nephron-Specific Knockout of NOS3

Doxycycline-induced nephron-specific NOS3 DNA recombination was assessed 4 weeks after doxycycline treatment in heart, lung, liver, stomach, intestine, testis, spleen, and kidney. Recombination of the NOS3 gene was detected in liver and kidney of NOS3 KO mice, consistent with the expected organ recombination pattern using the Pax8-rtTA/LC-1 system (Figure 1).[14,15]

Effect of Nephron NOS3 KO on Response to Chronic Salt Loading

At baseline (normal salt diet), NOS3 KO mice had a similar mean arterial pressure as control mice (except for day 6)

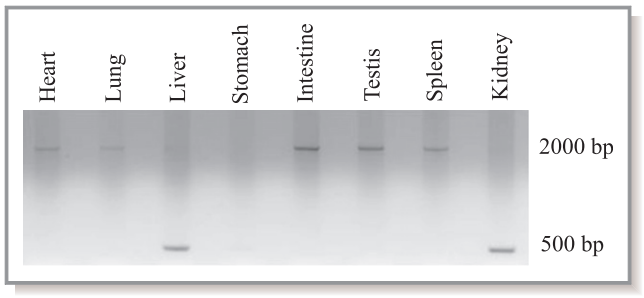

Figure 1. Representative PCR of NOS3 gene DNA recombination from organ panel from NOS3 KO mice (N=3).

(Figure 2A). Mean arterial pressure was also similar between the 2 genotypes during 7 days of low Na^+ diet (except for day 13). In contrast, NOS3 KO mice had significantly elevated BP compared with control mice on most days of high Na^+ diet. Pulse was similar between control and NOS3 KO mice when fed a normal, low or high Na^+ diet (Figure 2B). NOS3 KO mice had reduced urinary Na^+ excretion during the second day of salt loading compared with control mice (Figure 2C). Note that each day is shown as net Na^+ excretion since it is important to include Na^+ intake in determining Na^+ excretion. Plasma renin concentration was not significantly different between NOS3 KO and control mice after 7 days of low or high Na^+ diet, however PRC tended to be lower in NOS3 KO compared with control mice fed a high Na^+ diet (N=10, P=0.073) (Figure 3A). No differences in plasma Na^+, K^+, or GFR were detected between control and NOS3 KO mice at baseline and after 7 days of high Na^+ intake (Figures 3B through 3D). Urinary NOx excretion tended to be reduced in NOS3 KO mice at baseline (P=0.06), while NOS3 KO and control mice had similar urinary NOx excretion after 7 days of a high Na^+ diet (Figure 3E).

Effect of Nephron NOS3 KO on Response to Acute Salt Loading

To determine the effects of nephron NOS3 KO on renal salt handling during acute Na^+ loading, control and KO mice were given a 1.5-mL saline intraperitoneal injection. Urinary NOx excretion tended to be reduced in NOS3 KO as compared with control mice 4 hours after an acute salt load (P=0.09) (Figure 3F). No difference in plasma Na^+ or K^+ was detected between control and NOS3 KO mice after 4 hours of an acute salt load (Figures 3B and 3C). Control mice Na^+ excretion peaked at 4 hours after salt loading and was minimal at 6 and 7 hours; in contrast, NOS3 KO mice had comparatively delayed Na^+ excretion (Figure 4).

To help determine the nephron segment involved in the delayed Na^+ excretion following an acute salt load in NOS3 KO mice, furosemide (10 mg/kg), hydrochlorothiazide (15 mg/kg) or amiloride (5 mg/kg) were given to control and NOS3 KO mice along with saline (Figure 4). Furosemide caused a much more rapid natriuresis as compared with saline loading alone and abolished the difference in urinary Na^+ excretion between control and NOS3 KO mice following an acute salt load. Hydrochlorothiazide modestly increased the natriuretic response in both genotypes and reduced, but did not abolish, the differences in Na^+ excretion following an acute salt load between NOS3 KO and control mice. Amiloride had minimal effects on urinary Na^+ excretion (particularly in NOS3 KO mice) and did not alter the difference in the natriuretic response between NOS3 KO and control mice.

Effect of Nephron NOS3 KO on Total Kidney Protein Expression

Under baseline conditions, total kidney NOS1 and NOS3 protein abundances were similar between control and NOS3 KO mice. Total kidney ENaC-β was marginally higher in NOS3 KO compared with control mice at baseline (Figures 5 and 6). No differences were detected in protein levels of ENaC-α, ENaC-γ, NHE3, p-NHE3, total NCC, p-T^{53}-NCC (hereafter referred to as p-NCC), the ratio of p-NCC/total NCC, total NKCC2, p-$T^{96/101}$-NKCC2, or the ratio of p-$T^{96/101}$-NKCC2/total NKCC2 between NOS3 KO and control mice. Phospho-S^{126}-NKCC2 was undetectable at baseline in both genotypes. There were no differences between genotypes with regard to total SPAK (detects SPAK and OSR1), p-SPAK (detects both p-SPAK and p-OSR1), OSR1 (detects just OSR1), the ratio of p-SPAK/total SPAK or the ratio of p-SPAK/total OSR1.

High Na^+ intake for 1 week resulted in lower total kidney NOS3 protein abundance in NOS3 KO compared with control mice, while NOS1 abundance was not different between the two genotypes (Figures 5 and 6). ENaC-γ protein was marginally lower in NOS3 KO mice compared with control mice, however there were no differences in ENaC-α or ENaC-β between genotypes. NHE3 abundance was similar, while the percent phosphorylation of NHE3 was reduced, in NOS3 KO versus mice. High salt intake caused slightly reduced total but not phosphorylated NCC protein abundance in NOS3 KO compared with control mice resulting in an increased ratio of p-NCC/NCC in NOS3 KO compared with control mice. Both phosphorylated ($T^{96/101}$) and total NKCC2 were lower in NOS3 KO compared with control mice resulting in a similar ratio of p-$^{T96/101}$/total NKCC2 between the 2 genotypes. No p-S^{126}-NKCC2 was detectable in either genotype after 7 days of a high salt diet.

After an acute salt load (analyzed 4 hours post load), NOS3 protein expression was markedly lower in NOS3 KO compared with control mice, while NOS1 protein abundance was similar between the two genotypes (Figures 5 and 6). ENaC-α, but

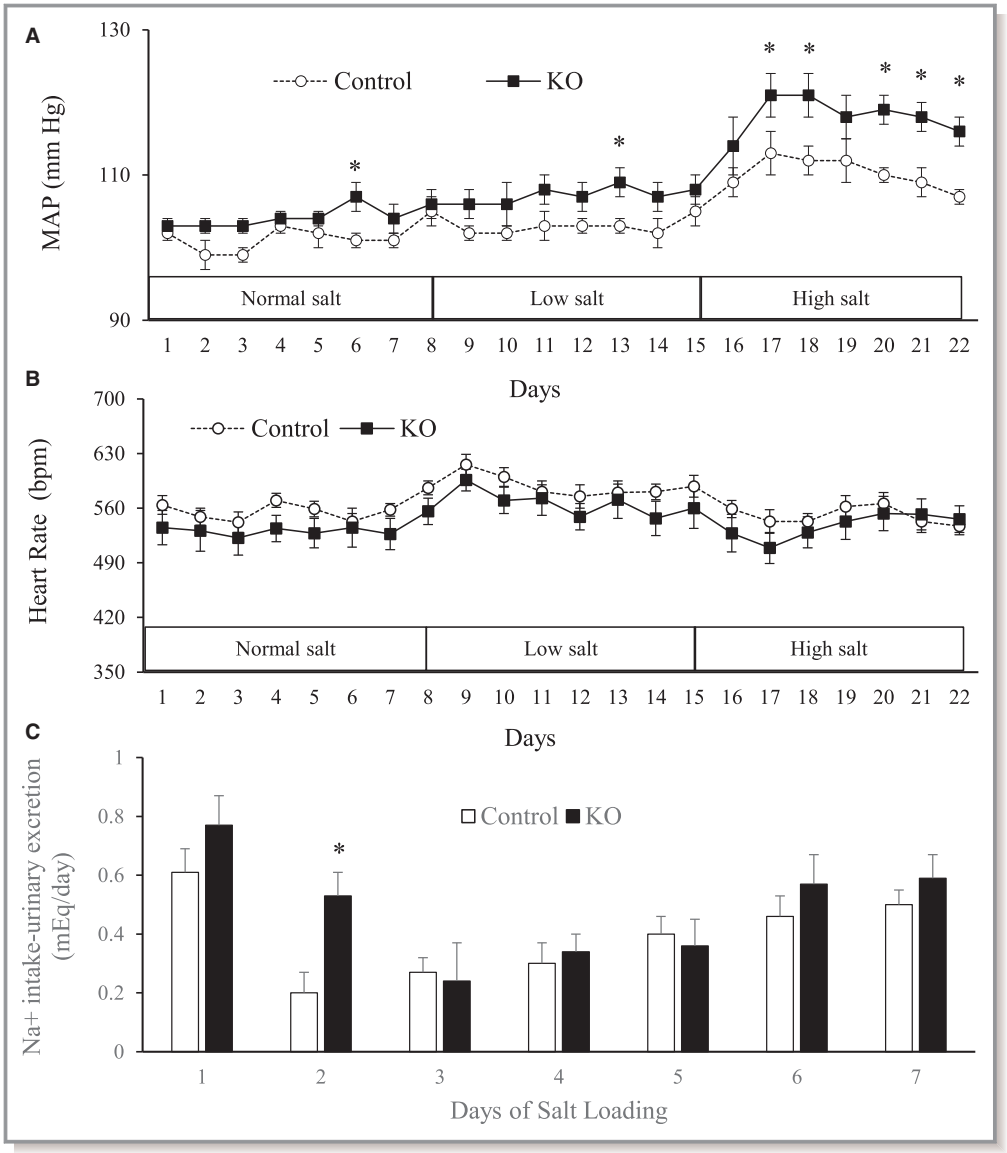

Figure 2. Mean arterial pressure (A), heart rate (B), and (C) daily (intake—urinary excretion) Na^+ balance in control and NOS3 knockout (KO) mice during normal, low and high Na^+ intake. N=6 to 10/data point (each mouse received a normal, then low, then high salt diet). *P<0.05 vs control mice.

not ENaC-β and ENaC-γ, protein levels were lower in NOS3 KO mice compared with control mice. No difference in phosphorylated or total NHE3 protein abundance between genotypes was evident. Both phosphorylated and total NCC were increased in NOS3 KO mice; the ratio of p-NCC/total NCC was reduced in NOS3 KO mice compared with controls. Acute salt loading was associated with higher p-$T^{96/101}$-NKCC2, but not total NKCC2, in NOS3 KO compared with control mice; this resulted in an increased ratio of p-$T^{96/101}$/total NKCC2. Acute salt loading also increased p-S^{126}-NKCC2 and the ratio of p-S^{126}/total NKCC2 in NOS3 KO compared with control mice. Acute salt loading did not change total SPAK or total OSR1 between the 2 genotypes, but it did cause higher p-SPAK in NOS3 KO compared with control mice; as a result, the ratios of p-SPAK/total SPAK and p-SPAK/total OSR1 were increased in NOS3 KO compared with control mice.

Discussion

A major finding in the current study is that nephron-wide NOS3 KO (hereafter NOS3 KO refers to nephron-wide NOS3 KO unless specifically stated otherwise) causes hypertension and impaired urinary Na^+ excretion during salt loading. The salt-sensitive BP finding is supported by previous studies wherein global NOS3 KO caused hypertension,[16,17] but re-expressing vascular endothelial NOS3 in the setting of global NOS3 KO did not normalize BP[18] suggesting that non-

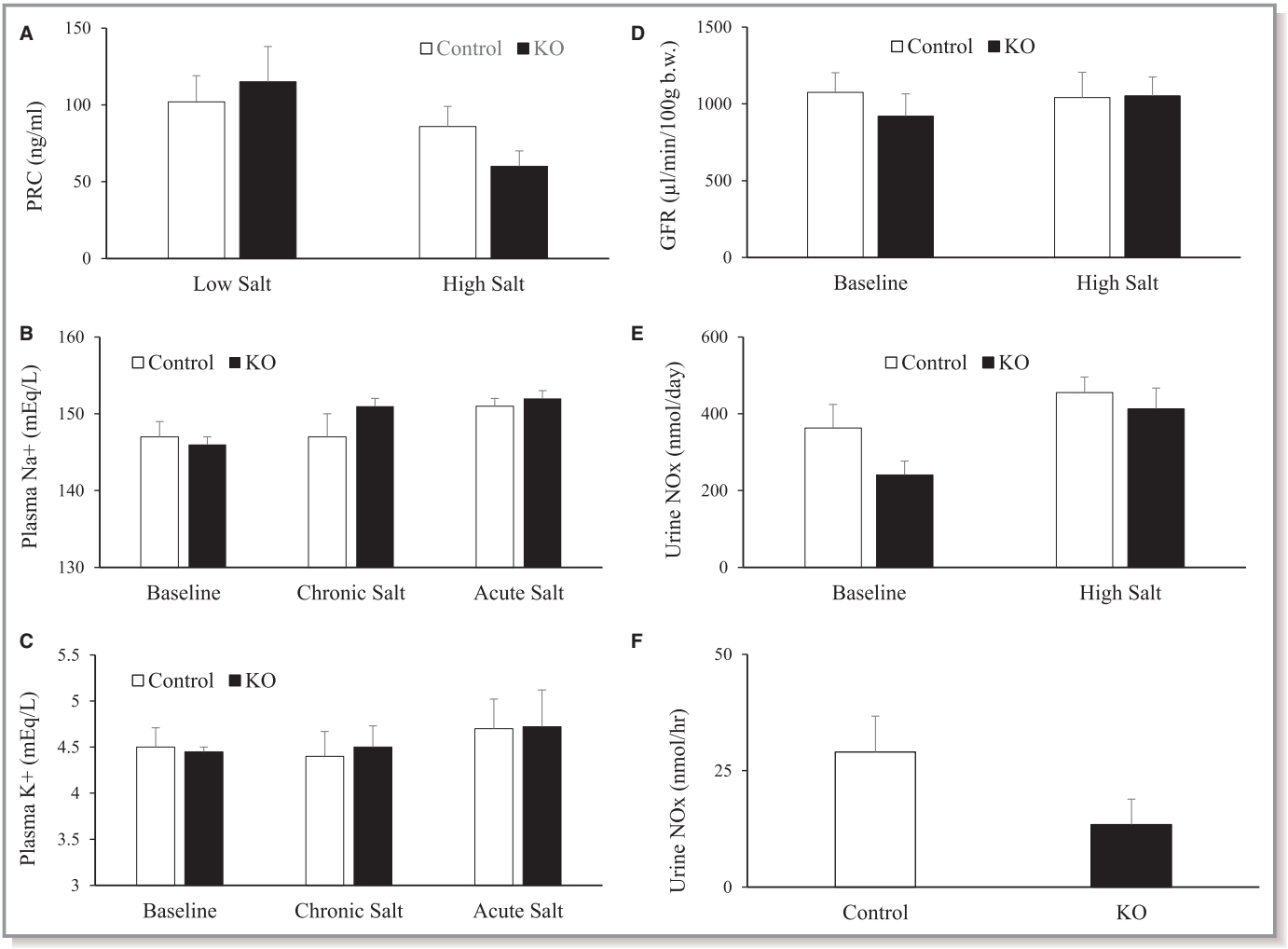

Figure 3. Plasma renin concentration (PRC) (A), plasma Na^+ (B) and K^+ (C) concentrations, glomerular filtration rate (GFR) (D), and urinary nitrate/nitrite (NOx) excretion after 7 days of normal or high salt intake (E) (N=12/data point except N=7/data point for GFR). Urine NOx after 4 hours of an acute salt load is shown in Panel F (N=6/data point). KO indicates knockout mice.

endothelial NOS3 modulates BP. Importantly, the hypertension and impaired Na^+ excretion in NOS3 KO mice was not associated with altered GFR. In addition, PRC tended to be suppressed in NOS3 KO mice. This likely relates to the hypertension although reduced NO stimulation of juxtaglomerular cell renin secretion cannot be excluded (ie, global NOS3 KO mice have reduced renal renin mRNA expression).[19,20]

We have previously demonstrated that normal kidneys do not have detectable nephron NOS3 immunostaining under baseline conditions or after salt loading.[8] Hence, it was not possible to determine the degree of nephron NOS3 protein knockdown beyond the demonstrated reduced whole kidney NOS3 in NOS3 KO mice (compared with control mice) during chronic and acute salt loading. No differences in NOS1 expression were observed between NOS3 KO and control mice under any conditions. This does not preclude a compensatory role of NOS1 in preventing or ameliorating decreased nephron NO production since changes in NOS1 activity could occur independently of total protein expression.

The current study suggests that nephron NOS3 may be primarily involved in regulating Na^+ excretion under relatively acute conditions. First, Na^+ retention was evident on day 2 during chronic salt loading in NOS3 KO mice and did not progressively increase (compared with controls) thereafter (day 1 of metabolic balance studies typical yields unreliable Na^+ excretion data attributable to the mice not eating or drinking as much as in the following days). Such failure to progressively increase Na^+ retention also likely relates to compensatory factors that re-establish Na^+ balance. Second, the acute salt loading studies showed delayed Na^+ excretion in NOS3 KO mice compared with controls. Third, the most pronounced differences in Na^+ transporter protein expression between NOS3 KO and control mice (discussed in detail below) were evident after acute salt loading. In agreement, a previous study showed high salt intake increased total NOS3

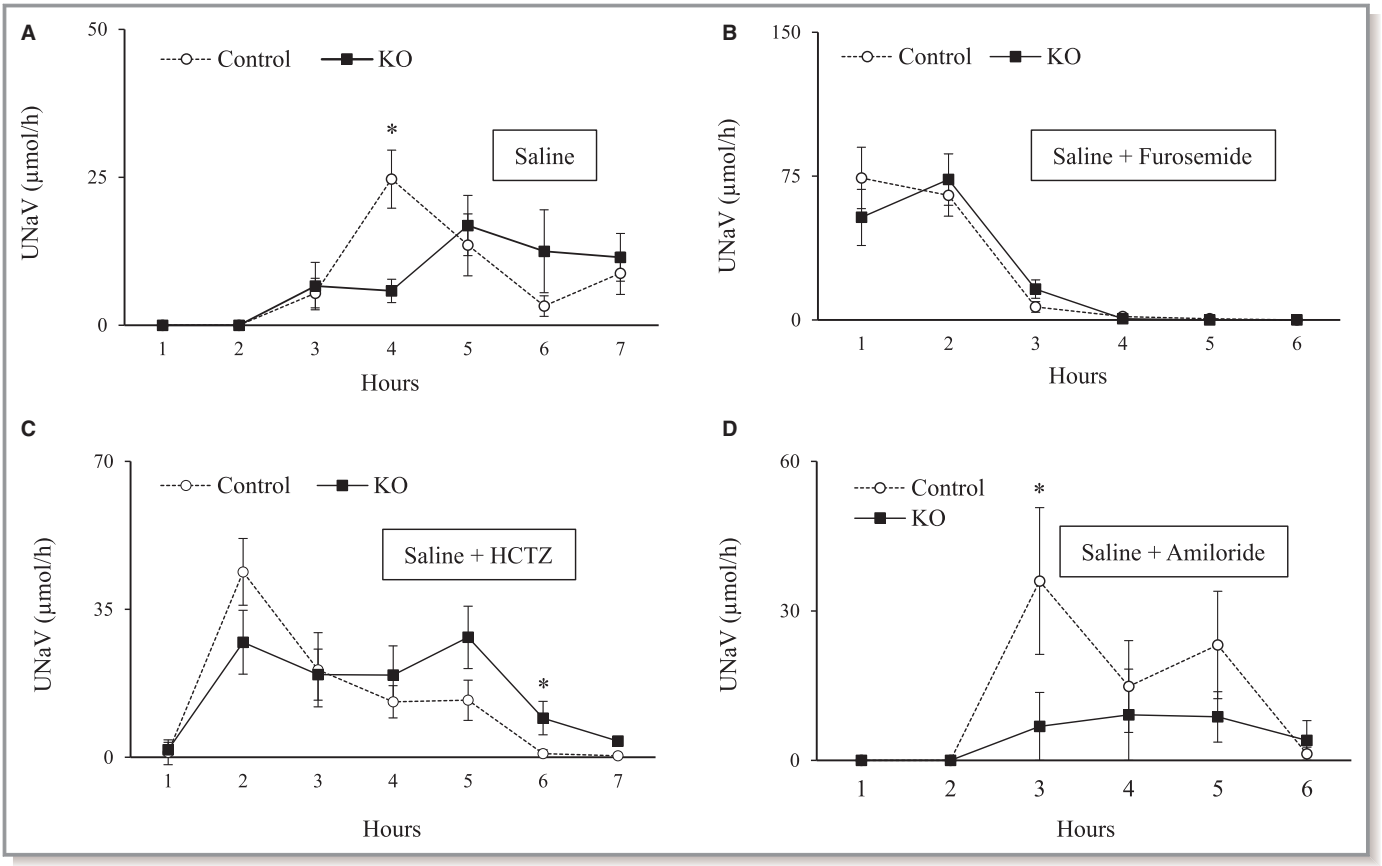

Figure 4. Hourly urinary Na^+ excretion in control and NOS3 knockout (KO) mice after 1.5-mL saline i.p injection (A) with or without furosemide (10 mg/kg) (B), hydrochlorothiazide (HCTZ, 15 mg/kg) (C) or amiloride (5 mg/kg) (D). N=6 to 12/data point. *P<0.05 vs control mice.

expression and activity in the thick ascending limb as early as 1 day (first measurement was at 1 day), while L-arginine-induced NO production in the thick ascending limb was increased by 1 day and normalized by 7 days after high salt intake.[21] This study also found that thick ascending limb basal and ET-1-stimulated NO production increased by 1 day and normalized by 3 days of a high salt diet and that this was mediated by NOS3.[21] Taken together, these findings point to nephron NOS3 playing a role in the short-term response to high salt intake. That said, NOS3 KO mice continue to have an elevated BP compare to controls after 7 days of high salt intake, indicating that NOS3 is also potentially involved in chronic BP regulation. The reasons for the sustained BP effect of NOS3 KO are unclear, however could involve, at least in part, continued NCC activation (see Discussion below).

It is unlikely that ENaC is involved in the hypertension or Na^+ retention observed in NOS3 KO mice. Small and variable (acute and chronic Na^+ loading) changes in ENaC-ß and -γ were observed. In contrast, ENaC-α was reduced with acute salt loading in NOS3 KO compared with control mice (please note that, despite trying several ENaC antibodies, we were unable to reproducibly detect cleaved ENaC-α or -γ). Further, amiloride did not prevent the delayed Na^+ excretion observed in NOS3 KO mice in response to an acute salt load. In agreement, and despite the finding that a high salt diet increased collecting duct NOS3 activity,[22] no effect of collecting duct-specific NOS3 KO on BP or urinary Na^+ excretion during normal or high salt intake was observed (ENaC expression was not measured in these studies).[8] In contrast, collecting duct-specific NOS1 KO caused salt-sensitive hypertension and Na^+ retention[3] as well as impaired inhibition of ENaC by ET-1.[4] Thus, it seems likely that collecting duct NOS1, but not NOS3, has a significant physiological role in modulating ENaC activity.

No differences in total or phosphorylated NHE3 were detected between NOS3 KO and control mice following acute or chronic salt loading with the exception of a small decrease in p-NHE3 during chronic salt loading. However, it is not possible to exclude a role for NHE3 in that we did not explore trafficking of NHE3 (known to be involved in the response to changes in BP and salt intake).[23] As alluded to in the Introduction, the role of NO in modulating proximal tubule NHE3 is uncertain in that both inhibitory and stimulatory responses have been reported.[6] Hence, while no changes in NHE3 protein were detected, further studies are needed to clearly resolve a potential role for NOS3 in modulating NHE3 activity and subcellular localization.

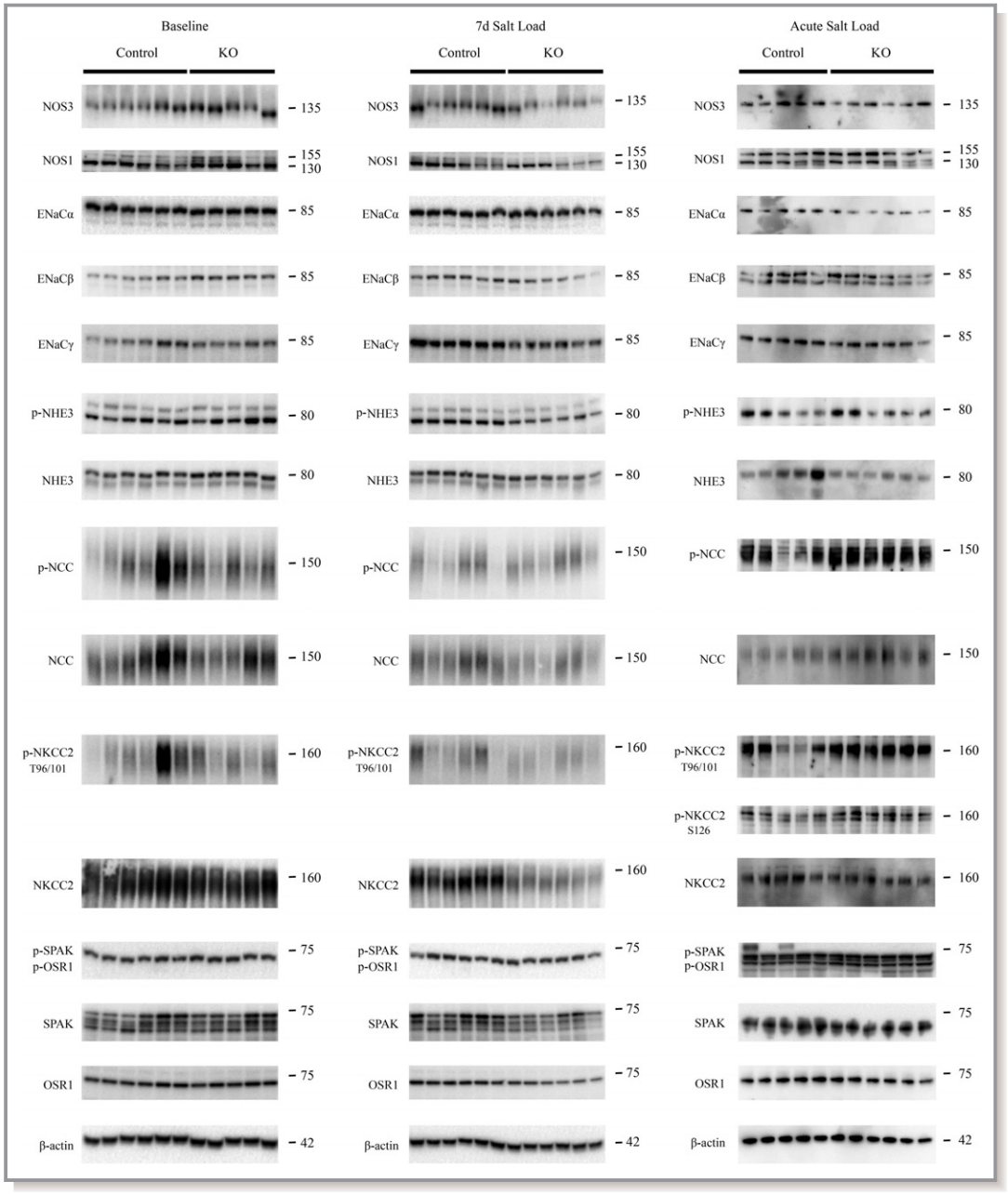

Figure 5. Western blots of transporter, channel and kinase proteins in control and NOS3 knockout (KO) mice fed a normal salt diet (left), 7 days of a high salt diet (middle) and after 4 hours of an acute salt load (right).

The current study's findings support a role for NCC in the delayed Na^+ excretion by NOS3 KO mice following acute salt loading. Total and p-NCC(T^{53}) were substantially higher in NOS3 KO compared with control mice following an acute salt load. Further, thiazide treatment appeared to partially mitigate the delay in Na^+ excretion by NOS3 KO mice following acute salt loading. In contrast, NCC levels were only modestly different between the two genotypes after a week of chronic salt intake and of uncertain biologic significance (p-NCC was not different, while total NCC was lower in NOS3 KO mice, giving a greater p-NCC/total NCC ratio). Speculatively, perhaps NOS3 KO hypertension suppresses total NCC but the absence of NOS3 continues to promote relatively greater phosphorylation of NCC. Overall, these findings are in agreement with a preliminary study reporting that L-NAME hypertension was reduced by hydrochlorothiazide, but not amiloride, and that L-NAME increased cultured mouse distal convoluted tubule NCC expression.[24]

NKCC2 also appears to be involved in the delayed Na^+ excretion by NOS3 KO mice following acute salt loading. While total NKCC2 was not different, both p-$T^{96/101}$-NKCC2 and p-S^{126}-NKCC2 were greater in NOS3 KO compared with control mice after an acute salt load. Additionally, furosemide abolished the difference in Na^+ excretion between the 2 genotypes following

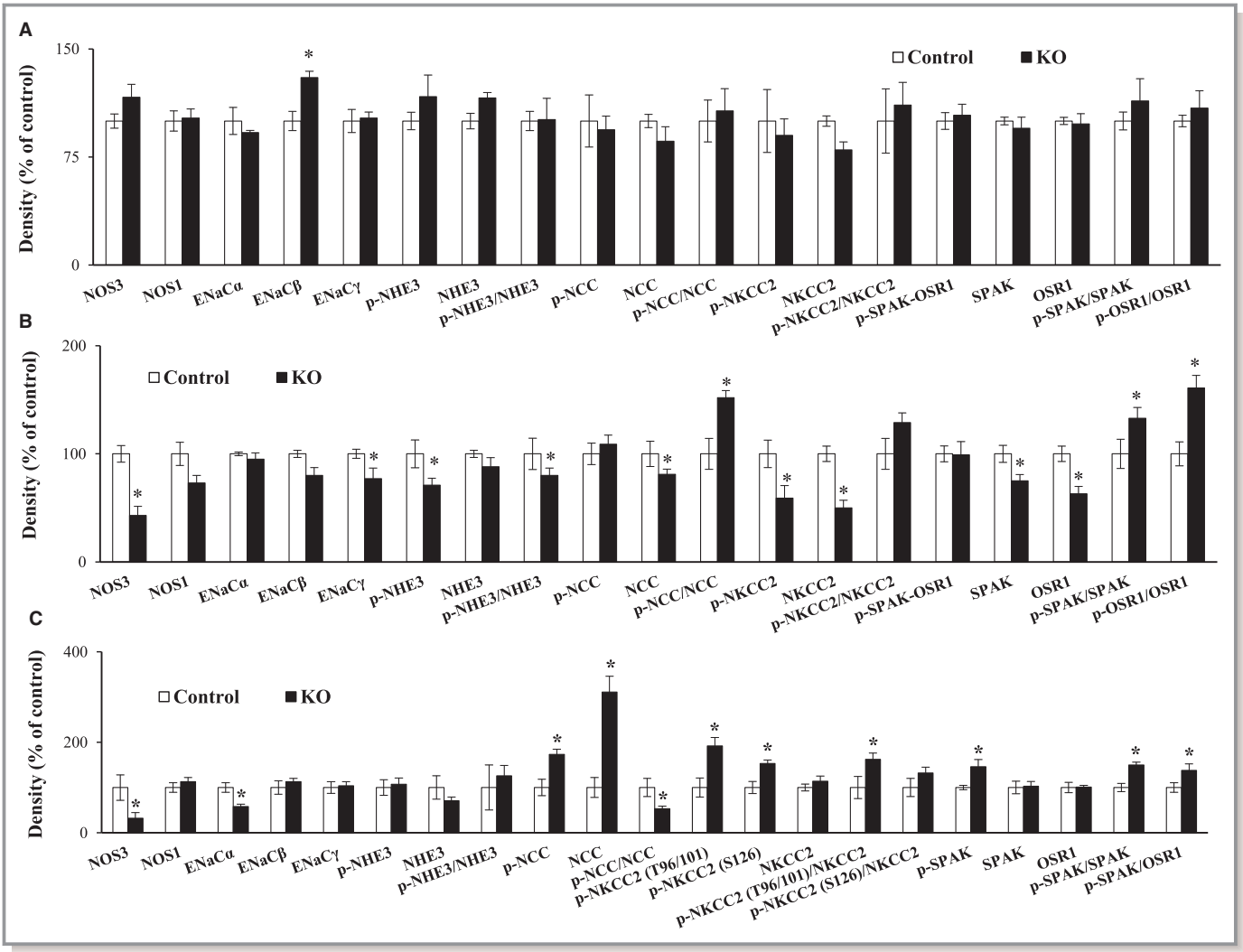

Figure 6. Relative densitometries of transporter, channel and kinase protein abundance in control and NOS3 knockout (KO) mice fed a normal salt diet (A), 7 days of a high salt diet (B) and after 4 hours of an acute salt load (C). N=5 to 6/data point. *P<0.05 vs control mice. Ratios of phosphorylated to total proteins are shown by "p-protein/protein".

acute salt loading. In contrast, total and p-$T^{96/101}$-NKCC2 were lower in NOS3 KO versus control mice after chronic salt loading (p-S^{126}-NKCC2 was undetectable except following an acute salt load). These findings are in agreement with several previous studies indicating that NO in general, and NOS3 in particular, inhibits thick ascending limb NKCC2. As mentioned earlier, NO, via cGMP, inhibits NKCC2 activity in isolated thick ascending limb[1,2]; thick ascending limb NO production, whether agonist-modulated (ET-1, angiotensin-II, or inflammatory cytokines)[12,25,26] or in disease states (diabetes mellitus and Dahl salt-sensitive hypertension[27,28]) derives, at least in part, from NOS3.

The current study found that SPAK/OSR1 is likely activated in NOS3 KO mice, particularly in the setting of acute salt loading. While total SPAK/OSR1 was not different between genotypes following acute salt loading, p-SPAK/OSR1 was higher in NOS3 KO versus control mice. In contrast, chronic salt intake was associated with slightly lower total SPAK/OSR1 in NOS3 KO compared with control mice while p-SPAK/OSR1 was not different between the 2 genotypes. Such increased SPAK/OSR1 phosphorylation potentially could be responsible, at least in part, for increased NCC and NKCC2 phosphorylation seen in the setting of acute salt loading. The current study found that, after acute salt loading, NOS3 KO mice had higher phosphorylation of NCC at threonine-53; this is a key NCC regulatory site and is known to be phosphorylated by SPAK/OSR1.[29,30] In addition, a preliminary study has found that knockout of WNK4 (which phosphorylates SPAK) reduces total and phosphorylated NCC.[31] Similarly, the current study found that NOS3 KO mice had higher phosphorylation of NKCC2 at threonines 96/101 after acute salt loading; this is a major NKCC2 regulatory site and is known to be phosphorylated by SPAK/OSR1.[32] Notably, NOS3 KO also increased NKCC2 phosphorylation

at S^{126}; Serine-126 is the only other NKCC2 phosphorylation site known to increase transporter activity; its phosphorylation is regulated by cAMP/protein kinase A, but not by SPAK/OSR1.[32] Thus, NOS3 may regulate NKCC2 through potentially multiple pathways.

While the current study provides evidence that NCC and NKCC2 are involved in the impaired natriuretic response to acute salt loading in NOS3 KO mice, additional studies could be done. One such study could involve attempts to analyze apical plasma membrane expression/distribution of NCC and NKCC2 using cell surface biotinylation or quantitative immunostaining; such studies could be extended to NHE3 and ENaC. In addition, immunostaining of SPAK/OSR1 might be performed to attempt to identify which cell types (thick ascending limb and distal convoluted tubule in particular) have altered SPAK/OSR1 expression and/or phosphorylation in NOS3 KO mice. Further, other transporters could be examined, including pendrin, the Na^+/K^+-ATPase, Na^+-dependent $Cl^-/HCO3^-$ exchanger, Na^+/glucose cotransporter, Na^+/P_i cotransporter and others. Other key questions include how NO regulates SPAK/OSR1 and what other mechanisms (independent of SPAK/OSR1) NO uses to modulate NCC and NKCC2 in vivo. In essence, the current study was designed to discover if NOS3 regulated nephron Na^+ transport and BP in vivo and then to begin to identify major mechanisms by which NOS3 might modulate such Na^+ transport. Clearly, this is an area that will need substantial additional studies.

In summary, this is the first study to: (1) disrupt NOS3 specifically within the entire nephron; (2) identify a role for nephron NOS3 in vivo in regulating renal Na^+ transport and BP; (3) describe potential NO regulation of NKCC2 in vivo; (4) show potential NO modulation of NCC; and (5) show potential NOS3 regulation of SPAK/OSR1. Beyond their physiological relevance, these findings are particularly important considering the findings that NOS3/NO regulation of NKCC2 may partly account for genetic salt-sensitive hypertension (Dahl S versus Dahl R rats),[33] angiotensin II-induced hypertension[12,25,26] and diabetes mellitus-associated hypertension.[27] Further, these findings suggest that NOS3-derived NO in the distal tubule may be important in BP regulation in health and disease, a heretofore unexplored area.

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Disclosures

None.

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