Renal Collecting Duct NOS1 Maintains Fluid–Electrolyte Homeostasis and Blood Pressure

Kelly A. Hyndman, Erika I. Boesen, Ahmed A. Elmarakby, Michael W. Brands, Paul Huang, Donald E. Kohan, David M. Pollock, Jennifer S. Pollock

Abstract—Nitric oxide is a pronatriuretic and prodiuretic factor. The highest renal NO synthase (NOS) activity is found in the inner medullary collecting duct. The collecting duct (CD) is the site of daily fine-tune regulation of sodium balance, and led us to hypothesize that a CD-specific deletion of NOS1 would result in an impaired ability to excrete a sodium load leading to a salt-sensitive blood pressure phenotype. We bred AQP2-CRE mice with NOS1 floxed mice to produce flox control and CD-specific NOS1 knockout (CDNOS1KO) littermates. CDs from CDNOS1KO mice produced 75% less nitrite, and urinary nitrite+nitrate (NOx) excretion was significantly blunted in the knockout genetope. When challenged with high dietary sodium, CDNOS1KO mice showed significantly reduced urine output, sodium, chloride, and NOx excretion, and increased mean arterial pressure relative to flox control mice. In humans, urinary NOx is a newly identified biomarker for the progression of hypertension. These findings reveal that NOS1 in the CD is critical in the regulation of fluid–electrolyte balance, and this new genetic model of CD NOS1 gene deletion will be a valuable tool to study salt-dependent blood pressure mechanisms. (Hypertension. 2013;62:91-98.) ● Online Data Supplement

Key Words: blood pressure • collecting duct • NOS1 • salt-sensitivity • sodium excretion

Understanding renal control of sodium excretion is not only important for management of fluid–electrolyte disorders, but perhaps as important, and is critical for elucidating defects in a wide array of diseases, such as hypertension and diabetes mellitus. Clinically, patients with hypertension have reduced urinary NO metabolite excretion (NOx, nitrite+nitrate, a measure of NO production) compared with age-matched normotensive patients. Furthermore, it was recently determined that urinary NOx excretion is a biomarker for predicting the progression of hypertension in normotensive men.

NO is produced during the conversion of L-arginine to L-citrulline by NO synthase (NOS). In the rat kidney, the inner medullary collecting duct (IMCD) has the highest total NOS activity as well as expressing NOS1 (neuronal NOS). The collecting duct (CD) is the site of daily fine-tune regulation of sodium balance, thus highlighting the importance of renal NOS1 signaling in blood pressure control. By using rodent models, the physiology and mechanisms of the renal NOS1NO system in the regulation of blood pressure can be elucidated.

The NOS1 gene is complex, with multiple promoters, first exons, and start codons, as shown in humans and rodents. At least 3 NOS1 protein splice variants are expressed in the rat kidney and mouse brain. These have been termed NOS1α (the full-length protein), NOS1β, and NOS1γ, which have truncated N termini compared with NOS1α. Purified NOS1β has ≈80% of the activity of NOS1α, whereas NOS1γ has only 3% of the activity. In the commercially available NOS1KO mouse, the NOS1 gene was targeted for deletion in exon 2, an exon expressed only in NOS1α (Figure S1 in the online-only Data Supplement). Surprisingly, this NOS1αKO mouse is normotensive, even on an HS diet. A total NOS1 gene knockout mouse was generated by targeting exon 6 for deletion, with exon 6 being conserved among the NOS1 splice variants. These mice have a severe phenotype, whereby they are infertile with hypogonadism, and only survive on a liquid diet. Currently, these mice have not been studied with regard to renal excretory function or blood pressure control.

Tissue- or cell-specific gene knockout mice using the Loxp-Cre system have been instrumental in our understanding of cell signaling. Aquaporin-2 (AQP2) is exclusively expressed in the renal collecting duct (CD) principal cells, vas deferens, and seminiferous tubules within testis. Studies using AQP2-Cre positive mice have been helpful in our understanding of renal CD physiology and pathophysiology. We hypothesized that deletion of all the NOS1 variants in the CD will
lead to a defect in salt handling and blood pressure regulation. To test our hypothesis, we generated CD principal cell NOS1 KO mice (CDNOS1KO) using the flox exon 6 NOS1/AQP2-Cre system. Deletion of NOS1 in the CD results in a rightward shift of the pressure–natriuresis relationship when challenged with an HS diet, thus displaying a salt-sensitive blood pressure phenotype. We have identified a novel and necessary physiological function for NOS1 in the CD in fluid–electrolyte homeostasis and blood pressure regulation.

Methods

All animal breeding, housing, and protocols were approved by the Institutional Animal Care and Use Committee in facilities accredited by American Association for the Accreditation of Laboratory Animal Care at Georgia Regents University.

Generation of the CDNOS1KO Mouse

NOS1αKO mice and wild-type (WT; C57BL/6J) control mice were purchased from Jackson Laboratories (Bar Harbor, ME). Transgenic mice were bred as previously described. In short, male homozygous NOS1 exon 6 flox mice were mated with hemizygous, AQP2-Cre female mice. Female offspring that were AQP2-Cre positive and heterozygous for NOS1 floxed transgene were then mated with homozygous NOS1 floxed male mice. Female offspring from this mating, which were homozygous for the flox NOS1 transgene and AQP2-Cre positive, were then mated with homozygous NOS1 floxed male mice to produce 50% homozygous NOS1 flox transgene/AQP2-Cre positive (ie, CDNOS1KO mouse) or only homozygous NOS1 flox transgene (flox control mouse). The genotyping protocol can be found in the online-only Data Supplement.

Tissue and Collecting Duct Isolation

Inner medullae and cerebella were dissected from WT and NOS1αKO mice for Western blotting analyses. IMCDs were isolated as previously described. Isolated CDs were immediately processed for Western blotting or incubated with Hank’s Balanced Salt solution (HBSS) + 250 μmol/L L-arginine + 200U/mL superoxide dismutase for 1 hour for nitrite measurements. Western blots were performed as previously described, and antibodies are listed in Table S1 in the online-only Data Supplement.

Dietary Salt Intervention

After weaning, animals were provided normal salt (NS; Na=0.3%, NaCl=0.4%) standard pellet chow (Teklad, Madison, WI). For histological analyses, mice were fed an NS or HS (Na=1.6%, NaCl=4.0%) standard pellet chow diet for 7 days (Teklad). For metabolic cage studies, to prevent pellet chow contamination of urine, mice were fed a gel diet (Micro stabilized Rodent Liquid Diet, TestDiet, Richmond, IN) plus 6.3% agar, with a NS (0.25 g NaCl/100 g food, thus, Na=0.12% or NaCl=0.24%) and water ad libitum. During the low-salt (LS) intervention, mice were fed the same gel diet, but with 0.005 g NaCl/100 g (Na=0.0125% or NaCl=0.005%) food for 7 days, and then changed to an HS diet (2.4 g NaCl/100 g food, thus Na=1.2% or NaCl=2.4%) and water ad libitum for 7 days. All experiments were performed on male animals, 10 to 16 weeks old, with an average mass of 28.8±0.73 g for the flox control mice and 29.2±1.1 g for the CDNOS1KO mice.

Blood Pressure and Metabolic Cages

Telemetry devices (Data Sciences, PA-C10, St. Paul, MN) were implanted into the left carotid artery of isoluraneanesthetized mice. Mice were allowed 10 days to recover before collection of telemetry data. For metabolic studies, mice were acclimatized in the cages for 3 days before measurements. Mice had access to water ad libitum, while they were in the metabolic cages. Urine was collected twice daily (12-hour collection periods) under sterile, water-saturated mineral oil, from mice on NS diet for 6 days, followed by LS diet for 6 days, and then 6 days of HS diet. Urine was centrifuged at 1000g for 5 minutes, aliquoted, and stored at −80°C until further analysis.

Urinary NOx, Albumin, Prostaglandin E Metabolite, Ion Excretion, and CD Nitrite Production

Nitrate and nitrate (NOx) concentration in urine and nitrite in the HBSS from the CD incubations was analyzed using the E-NO 20 HPLC system (Eicom, Kyoto, Japan) as previously described. Albumin concentration was determined by ELISA (Exocell, Philadelphia, PA). Urinary prostaglandin E (PGE) metabolites were measured by PGE metabolite–enzyme immunoassay following manufacturer instructions (Cayman Chemical, Ann Arbor, MI). Urinary potassium and chloride concentration rates were determined by ion selective electrode (Medica Easylyte, Bedford, MA), sodium by the Analyst 200 Atomic Absorption Spectrometer (Perkin Elmer, Waltham, MA), and osmolality by the Micro-Osmometer 3300 (Advanced Instruments, Norwood, MA).

Histology and Immunohistochemistry

Five kidney sections of 5 μm thickness per animal were analyzed as previously described. Kidney sections were stained with Gomori’s trichrome (Richard Allan Scientific, Kalamazoo, MI) or hematoxylin and eosin. An expert mouse renal pathologist, blinded to the

Table. Intake and Urinary Excretion Measurements From Flox (n=6–8, Unless Noted) and CDNOS1KO (n=6–8) Mice on a Low-Salt Diet (0.005% NaCl), Normal-Salt Diet (0.24% NaCl), or High-Salt Diet (2.4% NaCl) for 6 Days

<table>
<thead>
<tr>
<th>Dietary NaCl Parameter Genotype</th>
<th>Flox</th>
<th>CDNOS1KO</th>
<th>Flox</th>
<th>CDNOS1KO</th>
<th>Flox</th>
<th>CDNOS1KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water intake, mL/d</td>
<td>2.2±0.8</td>
<td>2.1±0.3</td>
<td>1.3±0.1</td>
<td>1.4±0.1</td>
<td>6.0±0.4*†</td>
<td>5.1±0.9*†</td>
</tr>
<tr>
<td>Food intake, mL/d</td>
<td>8.3±0.2</td>
<td>8.4±0.26</td>
<td>8.2±0.4</td>
<td>7.9±0.3</td>
<td>7.9±0.6</td>
<td>7.2±0.3</td>
</tr>
<tr>
<td>Sodium intake, mg/d</td>
<td>0.2±0.01</td>
<td>0.2±0.01</td>
<td>9.9±0.5*</td>
<td>9.5±0.4*</td>
<td>94.8±6.8*†</td>
<td>86.7±3.9*†</td>
</tr>
<tr>
<td>Urine production, mL/d</td>
<td>1.0±0.2</td>
<td>0.8±0.2</td>
<td>1.0±0.2</td>
<td>1.4±0.4</td>
<td>2.8±0.5*†</td>
<td>2.3±0.4*</td>
</tr>
<tr>
<td>Protein excretion, mg/d</td>
<td>2.0±0.1</td>
<td>1.4±0.3</td>
<td>1.2±0.2</td>
<td>0.9±0.1</td>
<td>1.4±0.9</td>
<td>1.4±0.5</td>
</tr>
<tr>
<td>Albumin excretion, μg/d (n=4)</td>
<td>ND</td>
<td>ND</td>
<td>24.0±5.6</td>
<td>26.7±5.2</td>
<td>35.1±4.7</td>
<td>36.1±1.4</td>
</tr>
<tr>
<td>Urine osmolality mOsm/kg H2O</td>
<td>3526±686</td>
<td>2632±423</td>
<td>3495±503</td>
<td>2847±257</td>
<td>2446±180</td>
<td>2750±427</td>
</tr>
<tr>
<td>Sodium excretion, mmol/d</td>
<td>0.05±0.01</td>
<td>0.05±0.02</td>
<td>0.3±0.06*</td>
<td>0.3±0.04*</td>
<td>1.5±0.2†</td>
<td>1.2±0.1†</td>
</tr>
<tr>
<td>Chloride excretion, mmol/d</td>
<td>0.2±0.04</td>
<td>0.2±0.05</td>
<td>0.5±0.04</td>
<td>0.5±0.05</td>
<td>1.5±0.2†</td>
<td>1.1±0.1†</td>
</tr>
<tr>
<td>Potassium excretion, mmol/d</td>
<td>0.2±0.02</td>
<td>0.1±0.03</td>
<td>0.2±0.02</td>
<td>0.2±0.03</td>
<td>0.2±0.03</td>
<td>0.2±0.04</td>
</tr>
</tbody>
</table>

No statistically significant differences between flox and CDNOS1KO mice. ND indicates not determined.

*P<0.05 for flox or CDNOS1KO compared with 0.005% NaCl diet, respectively.
†P<0.05 for flox or CDNOS1KO compared with 0.24% NaCl diet, respectively.
genotype of the mice, analyzed kidneys for renal structural anomalies. For immunohistochemical analyses, detection of NOS1, CD3, and F4/80 was accomplished by incubating the tissue sections overnight at 4°C (Table S1). Immunoreactivity was visualized with the Olympus BX40 microscope, affixed with an Olympus DP70 camera. Full-kidney section scans were obtained from a PathScan Enabler IV (Meyer Instruments, Houston, TX). Brightness and contrast of the images were adjusted with Microsoft PowerPoint (v. 12.1.5).

Statistics
All data are expressed as mean±SEM. When assessing the effect of diet on the flox and CDNOS1KO mice, 2-way ANOVA was performed (diet and genotype), followed by 1-factor ANOVA and Tukey post hoc test. Comparison of CD nitrite production or urinary NOx excretion on an NS diet was evaluated with unpaired, 2-tailed, Student t Test. P>0.05 was considered significant.

Results

NOS1 Expression
NOS1 splice variants are formed through alternative splicing of exon 2 (Figure S1). This alternative splicing results in truncation of the N terminus; however, the C termini are 100% identical. Thus, tissue and cellular distribution of these variants can be distinguished by using N terminus–specific NOS1 antibodies, which detect only NOS1α, versus C terminus–specific NOS1 antibodies, which detect all splice variants. To characterize NOS1 expression, inner medulla (IM) and cerebellum were homogenized and immunoblotted with C terminus anti-NOS1 antibody. Specificity of the C terminus NOS1 immunoreactivity was confirmed by peptide antigen blockade of the antibody (Figure S2). The WT mouse cerebellum homogenate expressed a predominant immunoreactive band at 155 kDa, which was not present in the cerebellum homogenate from the NOS1αKO mouse. In the homogenate from IM of WT mice as well as NOS1αKO mice, a NOS1 variant of 130 kDa was observed (Figure 1A). These bands correspond to NOS1α (155 kDa) and NOS1β (130 kDa). The 130-kDa band in the IM homogenate was distinct from the NOS3-specific band of 135 kDa (Figure 1B).

Finally, from a Western blot of freshly isolated IMCDs from WT and NOS1αKO mice, we found expression of the NOS1β splice variant exclusively (Figure 1C).

Confirmation of Deletion of NOS1 From the Collecting Duct
We isolated CDs from flox control and CDNOS1KO mice and found NOS1β expression in the flox control mice, but none in the CDNOS1KO mice (Figure 2A). Moreover, freshly isolated IMCDs from CDNOS1KO mice on an NS diet produced ≈75% less nitrite than flox control mice (P=0.003; n=3; Figure 2B), and CDNOS1KO mice excreted ≈50% nitrite/nitrate (NOx) compared with flox mice (P<0.01; n=13; Figure 2C).

NOS1 gene deletion from the CD was also confirmed by immunohistochemistry. As shown in Figure 2D, NOS1 positive immunostaining is apparent in the macula densa and vasa recta from both flox control and CDNOS1KO mice. In addition, we observed positive NOS1 immunostaining in cortical CDs, outer medullary CDs, and IMCD in flox control with no apparent staining in cortical CDs, outer medullary CDs, and IMCDs in CDNOS1KO mice (Figure 2D).

Effects of CD NOS1 Deletion on the Kidney
No obvious renal structural pathologies were noted in the CDNOS1KO mice, and papilla development was normal (Figure 3A–3C). Glomeruli and tubules all seemed normal, and indistinguishable between the 2 genotypes (Figure 2C). Because renal inflammation is involved in the pathogenesis of a variety of renal diseases, including salt-sensitive hypertension,20 we determined the number of CD3+ cells (T-cell marker) and F4/80+ cells (macrophage marker) in the renal cortex, outer and IM of each genotype. There were similar numbers of CD3+ immunoreactive cells (Figure 3D) and F4/80+ immunoreactive cells (Figure 3E) in the cortex and outer medulla between the flox and CDNOS1KO mice. No CD3+ or F4/80+ cells were found in the IM of either genotype (Figure 3D and 3E).

CDNOS1KO Mice Display a Salt-Sensitive Blood Pressure Phenotype
MAP, systolic blood pressure (SBP), and diastolic blood pressure (DBP) were determined by telemetry in flox control (n=9) and CDNOS1KO mice (n=11). While on an LS diet, CDNOS1KO and flox control mice had similar MAP, SBP, and DBP (Figure 4A; Figure S3A and S3B). When the flox control mice were placed on an HS diet, there were no significant changes in MAP, SBP, and DBP (Figure 4A; Figure S3A and S3B). The CDNOS1KO mice on an HS diet had significantly higher MAP, SBP, and DBP compared with flox control mice on an HS diet (Figure 4A; Figure S3A and S3B). When the CDNOS1KO mice were changed from an LS diet to an HS diet, MAP and SBP significantly increased and remained elevated for the 6 days of HS diet compared with LS diet (2-way ANOVA MAP: genotype P<0.16, diet P<0.009, interaction P=0.006; 2-Way ANOVA SBP: genotype P=0.04, diet P<0.02, interaction P=0.02; Figure 4A; Figure S3A). After 6 days of HS diet, DBP was significantly increased in CDNOS1KO mice compared with LS diet (2-Way ANOVA DBP: genotype P=0.89, diet P=0.61, interaction
$P=0.03$; Figure S3B). Heart rates were similar between the genotypes, and heart rate was not significantly affected by dietary sodium (Figure S3C). Pulse pressure was also not significantly different between the genotypes during LS or HS diet interventions (Figure S3D). However, it is notable that the CDNOS1KO mice have a pulse pressure >40 mm Hg during HS feeding (average over the diet interventions: LS=39±0.3 mm Hg, HS=42±0.3 mm Hg; Figure S3D).

**Deletion of CDNOS1 Results in Sodium Retention**

In a separate group of mice (n=6/genotype), urine volume, urinary sodium, chloride, and potassium excretion were determined. On an LS diet, CDNOS1KO and flox control mice excreted similar amounts of sodium (Figure 4B), chloride (Figure 4C), potassium (Figure 4D), and urine (Figure 4E). In addition, while on the LS diet, CDNOS1KO and flox control mice ate the same amount of food and drank similar amounts of water (Figure S4). Similarly, during the 6 days of HS diet, both flox control and CDNOS1KO mice ate similar amounts of food and drank comparable amounts of water (Figure S4). However, during the first 3 days of HS diet CDNOS1KO mice excreted significantly less sodium and chloride when compared with the flox control mice (Figure 4B and 4C). On day 4 and continuing to day 6 of the HS diet, both genotypes excreted similar amounts of sodium and chloride (2-way ANOVA sodium: genotype $P<0.004$, diet $P<0.001$, interaction $P=0.917$; 2-way ANOVA chloride: genotype $P<0.036$, diet $P<0.001$, interaction $P=0.92$; Figure 4B and 4C). Potassium excretion was similar between the genotypes on LS and HS diets, and increased significantly after 1 day of HS diet (Figure 4D; 2-way ANOVA potassium: genotype $P=0.26$, diet $P<0.001$, interaction $P=0.99$). Urine volume was similar in both genotypes on LS and HS diets, although reduced during the first day of HS diet in the CDNOS1KO mouse compared with the flox control mice (Figure 4B and 4C). On day 4 and continuing to day 6 of the HS diet, both genotypes excreted similar amounts of sodium and chloride (2-way ANOVA sodium: genotype $P<0.004$, diet $P<0.001$, interaction $P=0.917$; 2-way ANOVA chloride: genotype $P<0.036$, diet $P<0.001$, interaction $P=0.92$; Figure 4B and 4C). Potassium excretion was similar between the genotypes on LS and HS diets, and increased significantly after 1 day of HS diet (Figure 4D; 2-way ANOVA potassium: genotype $P=0.26$, diet $P<0.001$, interaction $P=0.99$). Urine volume was similar in both genotypes on LS and HS diets, although reduced during the first day of HS diet in the CDNOS1KO mouse compared with the flox control mice (2-way ANOVA volume: genotype $P<0.005$, diet $P<0.001$, interaction $P=0.98$; Figure 4E). Creatinine, protein, albumin, and electrolyte excretion rates as well as urine osmolality were similar between the 2 genotypes on LS diet, NS diet, and day 6 of HS diet (Table).

**CDNOS1KO Mice Display a Rightward Shift in the Pressure–Natriuresis Relationship**

We generated a pressure–natriuresis relationship from the MAP and sodium excretion data. CDNOS1KO mice display
a significant rightward shift in the pressure–natriuresis relationship compared with flox control mice (Figure 5A). This rightward shift in the curve is driven by a significant increase in MAP experienced by the CDNOS1KO mice on HS diet (Figure 5B; P=0.02).

Figure 4. When changed from a low-salt (LS) to high-salt (HS) diet, CDNOS1KO mice had a significant increase in mean arterial pressure compared with flox mice (n=8–11; A). B, For 3 days after the change to HS diet, CDNOS1KO mice excrete less sodium and (C) less chloride (n=8). D, Potassium excretion was similar between the genotypes (n=6). E, Urine excretion was significantly blunted in the CDNOS1KO mouse after 1 day of HS diet (n=8). †P<0.05 CDNOS1KO compared with Flox. *P<0.05 HS compared with LS.

Figure 5. Pressure–natriuresis relationship and the change in mean arterial pressure between a low-salt (LS) and 6-day high-salt diet (HS). A, There was a rightward shift in the pressure–natriuresis relationship in CDNOS1KO mice (closed symbols, solid) compared with flox control mice (open symbol, dashed line) on day 6 of HS diet intervention. This was driven by (B) a significant change in mean arterial pressure (MAP) from LS to day 6 HS diet in the CDNOS1KO mice. *P=0.02; n=8 to 11.

Figure 6. When challenged with high-salt diet for 1 day (HS1) or 7 days (HS7), CDNOS1KO mice have (A) blunted NOx excretion compared with flox control mice (n=6). B, Urinary PGE metabolite excretion was similar between the genotypes, but significantly higher after 1 day of high-salt diet (n=6). †P<0.05 CDNOS1KO compared with Flox. *P<0.05 HS compared with low salt (LS).

CDNOS1KO Mice Have Blunted HS-Induced NOx Excretion

Although CDNOS1KO and flox control mice on LS diet excrete comparable amounts of NOx (Figure 6A), when the diet is changed to HS, the NOx excretion significantly increased only in the flox control mice (P<0.05; n=8–10). After 6 days of HS diet, both flox control and CDNOS1KO mice had significantly higher NOx excretion compared with that on LS diet; however, the NOx excretion was significantly blunted in the CDNOS1KO mice compared with the flox control mice (2-way ANOVA NOx: genotype P<0.001, diet P<0.001, interaction P=0.09; Figure 6A). Flox control and CDNOS1KO mice excreted similar levels of PGE metabolites on LS diet, and this was significantly increased after 1 day of HS diet without any differences between the genotypes (2-way ANOVA: genotype P<0.24, diet P<0.001, interaction P=0.64; Figure 6B).

Discussion

The major finding from this study is that deletion of NOS1, specifically from the principal cells of CDs, results in an impaired ability to handle a salt load with a rightward shift in the pressure–natriuresis relationship and a salt-sensitive blood pressure phenotype. These data elucidated the functional relevance of NOS1-derived NO production in the CD to maintain sodium homeostasis and blood pressure control. We demonstrated that the mouse CD does not express NOS1α, but exclusively expresses NOS1β. We show that NOS1β expression is present in the CD of the commercially available NOS1α KO mouse, providing an explanation for the lack of a salt-sensitive phenotype in these mice.16 This study now clarifies the controversial role of NOS1 regulation of blood pressure.

Humans have multiple NOS1 splice variants,9–11 and NOS1 single nucleotide polymorphisms have been associated with hypertension.30,31 Humans express comparatively high levels of NOS1α in the cortex and outer medulla, with relatively less NOS1α expression observed in the IMCD,32 as determined by immunohistochemistry and Western blots with an N terminus NOS1-specific antibody that only detects NOS1α. Further analysis with quantitative reverse transcriptase polymerase chain reaction using primers designed to amplify between exons 17 and 20, which would be a measure of all NOS1 splice variants, showed significant expression of NOS1 in the
human renal cortex and medulla. These data suggest that renal NOS1 expression in humans may be similar to that in mice when there is a lower expression of NOS1α in the renal medulla. However, further work with kidney samples of normal humans and patients with hypertension is needed to fully characterize and distinguish NOS1 splice variant expression in the human kidney.

Much of the work related to NOS1 in the kidney has used rat models. Most studies used N terminus–specific antibodies, thus not allowing for a complete analysis of the NOS1 splice variants. Although, recently, Lu et al33 reported that all NOS1 splice variants are expressed in the rat macula densa, and that these are differentially regulated by dietary sodium, NOS1α was downregulated after a 10-day HS diet, whereas NOS1β was induced. In agreement with this finding, rats on an HS diet had an increase in macula densa–derived NO (presumably NOS1β–mediated) that attenuated the tubuloglomerular feedback mechanism. Similar to Lu et al,33 we recently reported that Sprague Dawley rats on an HS diet have a significant increase in IMCD NOS1β expression; however, IMCD NO production was not enhanced. However, HS diet significantly increased NO production in IMCD from WT mice without a change in NOS1β expression. Taken together, although there is distinct regulation of NOS1 splice variants between mice and rats, the commonality is that HS feeding is a physiological regulator of NOS1β in the kidney.

The NOS1 gene deletion in CDNOS1KO mice resulted in significantly less nitrite/nitrate (NOx) excretion compared with flox control animals. Although a majority of NOx in the body is derived from the diet (eating foods enriched in nitrite and nitrate), another source is the oxidation of NO. We found that CDNOS1KO mice ate similar amounts of food as the flox control mice, thus indicating that changes in urinary NOx most likely reflect changes in CD-derived NO production. In agreement with this finding, others have suggested that urinary NOx is reflective of renal NO production in both mice and humans. Likewise, our data indicate that a significant portion (≈50%) of urinary NOx is actually derived from the CD and from NOS1. Mattson’s laboratory found that the rat IMCD was the major source of NOS activity in the kidney; therefore, we measured IMCD nitrate production and found that the loss of NOS1 results in ≈75% less nitrite than control. This finding supports our hypothesis that urinary NOx excretion is, in part, a measure of CD-derived NO production. For humans, urinary NOx excretion has recently been identified as a diagnostic marker for predicting the progression of hypertension, with patients with hypertension excreting significantly less NOx than normotensive-matched controls.

One of the important mechanistic insights of this study was revealed when the mice were challenged with an HS diet. An HS challenge resulted in reduced sodium, chloride, NOx, and urine excretion with an increase in blood pressure in CDNOS1KO mice despite similar food and water intake compared with flox controls. The Guyton model of long-term control of arterial pressure predicts that the kidney alters sodium and water excretion to maintain fluid balance and arterial pressure, and is commonly referred to as the pressure–natriuresis/diuresis relationship(s). Previous work has suggested that the NO pathway modulates the pressure–natriuresis relationship, although little was understood about the cellular source or NOS isoform mediating this response. The current study defines CD NOS1 as a mediator of pressure–natriuresis/diuresis, because CDNOS1KO mice display a significant rightward shift in the pressure–natriuresis relationship, even when sodium excretion returned to comparable levels with the flox control mice.

The increase in MAP experienced by CDNOS1KO mice on an HS diet was largely driven by a significant increase in systolic pressure. Diastolic pressure was shown to be significantly different on day 6 of the HS diet. Consequently, we found that pulse pressure was not significantly different between flox and CDNOS1KO mice on either an LS or an HS diet. Although, CDNOS1KO mice did have a pulse pressure increase of ≈4 mm Hg on an HS diet compared with that on an LS diet. In clinical studies, pulse pressure is used as a predictor for cardiovascular disease, where differences as little as 4 mm Hg in pulse pressure have been shown to lead to increases in mortality. Salt-sensitivity (clinically defined as a >10 mm Hg drop in blood pressure attributable to sodium restriction for 24 hours) and pulse pressure were associated with an increase in mortality in both normotensive humans and patients with hypertension, especially for patients >60 years of age. Given these clinical studies and the finding that young CDNOS1KO male mice on an HS diet have a slightly elevated pulse pressure, but a significant elevation in systolic pressure, we predict that as CDNOS1KO mice age and consume an HS diet, they will experience increases in pulse pressure, vessel remodeling, decreases in baroreflex sensitivity and increases in mortality, as experienced by humans. Future research will test this possible hypothesis.

The CD produces PGE, a natriuretic and diuretic factor, and PGE metabolite excretion is increased with HS diet. We found that HS diet increased PGE metabolite excretion similar to CDNOS1KO and flox control mice. This suggests that the reduced sodium and chloride excretion observed in the CDNOS1KO mouse was not attributable to a significant reduction in PGE excretion and indicates that CD-derived NO does not regulate PGE excretion. We propose that flox control mice maintained sodium homeostasis through a direct tubular action of NOS1-dependent inhibition of sodium and water reabsorption, independent of PGE, although we cannot rule out that CD NOS1–derived NO regulates vasa recta resistance and medullary blood flow.

The importance of the CD in final control of sodium excretion and blood pressure regulation has been elaborated in previous studies with the use of CD-specific knockout mice, especially with that of the endothelin (ET-1) system that may be the upstream regulator of NOS1 activity. The CD is one of the major sources of renal ET-1 as well as renal NO activity. ET-1 via the ET B receptor (ET B) stimulates mouse CD NOS1–derived NO production, and previous reports suggested that CD ET B receptor signaling via NO production mediates ET-1–dependent control of sodium excretion. Both CDET1KO and CDET BKO mice display salt-sensitive hypertension that is similar to the CDNOS1KO mouse. Interestingly, CDET1KO
mice demonstrate attenuated NOx excretion in response to increased pressure or an HS challenge. ET-1 via the ET_{A} receptor decreases the open probability of CD epithelial sodium channels via direct tubular actions, although it is unknown whether this is via the NOS1/NO pathway. Therefore, future experiments will determine whether the HS-induced increase in blood pressure in CDNOS1KO mice is via the loss of the CD ET-1/ET_{A}/CD epithelial sodium channels signaling pathway.

Perspectives

CD NOS1 is a novel pathway involved in fluid–electrolyte balance and has received little attention within this complex regulatory system. Evidence for the importance of renal NO pathways to human physiology recently came from Baumann et al., who reported that urinary NOx is a biomarker for predicting the progression of hypertension in 75% of normotensive patients during a 4- to 5-year period. Moreover, patients with hypertension have reduced NOx excretion. 1-3 CDNOS1KO mice display a significantly low level of urinary NOx excretion with increased salt consumption leading to a salt-sensitive blood pressure phenotype. Thus, NOS1-derived NO in the CD regulates fluid–electrolyte balance and blood pressure. These basic studies highlight the importance of the CD NOS1 pathway and the necessity to translate these mechanistic findings to humans. The CDNOS1KO mouse model will facilitate testing future hypotheses focused on the NOS1-dependent mechanisms of cardiovascular and renal consequences of salt-sensitive hypertension.

Acknowledgments

We thank Dr Tatsuo Yamamoto (Second Department of Medicine, Numazu City Hospital, Numazu, Japan) for his renal pathology expertise. We appreciate the outstanding technical support from Janet Hobbs, Jackie Musall, Amy Dukes, and Melissa Durley-Giles.

Sources of Funding

This work was supported in part by the National Institutes of Health (HL06053 to J.S. Pollock); Cardiovascular Discovery Institute (to D.M. Pollock); and the National Kidney Foundation Post Doctoral Fellowship (to K.A. Hyndman). This work was also supported by the NIH Program Project Grant on Etdothenol Control of Renal Excretory and Hemodynamic Function (HL95499; to D.M. Pollock, J.S. Pollock, and D.E. Kohan).

Disclosures

None.

References


Renal Collecting Duct NOS1 Maintains Fluid–Electrolyte Homeostasis and Blood Pressure
Kelly A. Hyndman, Erika I. Boesen, Ahmed A. Elmarakby, Michael W. Brands, Paul Huang, Donald E. Kohan, David M. Pollock and Jennifer S. Pollock

Hypertension. 2013;62:91-98; originally published online April 22, 2013;
doi: 10.1161/HYPERTENSIONAHA.113.01291
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://hyper.ahajournals.org/content/62/1/91

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2013/04/22/HYPERTENSIONAHA.113.01291.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
ONLINE SUPPLEMENT

Renal Collecting Duct NOS1 Maintains Fluid-Electrolyte Homeostasis and Blood Pressure

Kelly A. Hyndman*, Erika I. Boesen†, Ahmed A. Elmarakby*/†, Michael W. Brands‡, Paul Huang‡, Donald E. Kohan§, David M. Pollock*†, Jennifer S. Pollock*†.

*Section of Experimental Medicine, Department of Medicine, †Department of Physiology, ‡Department of Oral Biology, Georgia Regents University, Augusta, GA 30912

‡Cardiology Division and Cardiovascular Research Center, Massachusetts General Hospital, Charlestown, MA 02129

§Division of Nephrology, University of Utah Health Sciences Center, Salt Lake City, UT 84132

Corresponding Author:
Jennifer S. Pollock, PhD
Section of Experimental Medicine, CB2200
Department of Medicine
Georgia Regents University
1459 Laney Walker Blvd
Augusta, GA, 30912
Telephone: 706 721 8514
Fax: 706 721 7661
Email: jpollock@georgiahealth.edu
Detailed Methods

**Genotyping**

DNA was extracted from mouse tail snips using MasterPure DNA Purification Kit (Epicentre Biotechnologies, Madison WI) and probed for the presence or absence of the AQP2-CRE transgene with the following primers: mAQP2 F: 5'- CTC TGC AGG AAC TGG TGC TGG -3', creTag R: 5'- GCG AAC ATC TTC AGG TTC TGC GG -3'. For the determination of the wild type or mutant (flox) NOS1, two PCRs were run with the following primers: Int5-1: 5'- GAC GTG TCT GCA ACT TCA GC -3', and 3.5B: 5'- GAT ACG TGT AGA GGG CAA ATG -3'. Or the INT5-1 forward with XK-U: 5'- CTA GGA AGG GGT CGG TAC -3' as the reverse. Wild type NOS1 gene results in a single band of 1.47 kbp. Heterozygous expression of the flox NOS1 results in a band of 1.47 and 1.4 kbp in the first PCR. Finally, a homozygous flox NOS1 results in a 1.47 kbp band in the first PCR and a 1.4 kbp band in the second PCR. Mice that were positive for AQP2-CRE and homozygous for flox NOS1 are the CDNOS1KO. Mice that were negative for AQP2-CRE and homozygous for flox NOS1 are the flox control mice.

Table S1: Antibodies used in the study.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Sequence</th>
<th>Amino Acids</th>
<th>Host</th>
<th>Application</th>
<th>Concentration</th>
<th>Company</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOS1</td>
<td>rat</td>
<td>C-terminus</td>
<td>rabbit polyclonal</td>
<td>IB</td>
<td>20 μg/10ml</td>
<td>Santa Cruz</td>
<td>Santa Cruz, CA</td>
</tr>
<tr>
<td>NOS1</td>
<td>human</td>
<td>1414-1434</td>
<td>rabbit polyclonal</td>
<td>IB, IHC</td>
<td>1.4 μg</td>
<td>Biomol</td>
<td>Plymouth Meeting, PA</td>
</tr>
<tr>
<td>NOS3</td>
<td>human</td>
<td>1025-1203</td>
<td>Mouse monoclonal</td>
<td>IB</td>
<td>12.5 μg/10ml</td>
<td>BD Biosciences</td>
<td>San Jose, CA</td>
</tr>
<tr>
<td>CD3-e</td>
<td>mouse</td>
<td>C-terminus</td>
<td>goat polyclonal</td>
<td>IHC</td>
<td>0.2 μg</td>
<td>Santa Cruz</td>
<td>Santa Cruz, CA</td>
</tr>
<tr>
<td>F4/80</td>
<td>mouse</td>
<td>?</td>
<td>rat monoclonal</td>
<td>IHC</td>
<td>0.1 μg</td>
<td>AbD Serotech</td>
<td>Raleigh, NC</td>
</tr>
</tbody>
</table>

IB- immunoblot  
IHC - immunohistochemistry
Figure S1. Diagram of the NOS1 splice variant (A) mRNA and (B) protein. A) The first 7 exons of the mouse NOS1 splice variants with predicted start sites (ATG) listed. The dotted line represents the spliced exons. Represents the loxP sites inserted to flank exon 6. X represents where the neomycin cassette was inserted using homologous recombination to delete exon 2 in the NOS1α knockout mouse from Jackson Laboratories (Huang et al. 1993). B) The predicted protein structure of the NOS1 splice variants. NOS1β and NOS1γ lack the PDZ domain that is encoded by exon 2, and form N-truncated proteins. The approximate location of the N-terminus and C-terminus antibodies used in our studies are indicated. Note this diagram is drawn to approximate size. Adapted from Eliasson et al. 1997.

Figure S2. Representative immunoblot (IB) of 10 µg of inner medullary (IM) or cerebellar (CB) homogenate from a wild type mouse. The left blot was incubated in C-terminus anti-NOS1 preincubated with the peptide antigen. The right blot was incubated in C-terminus anti-NOS1 and note that the peptide blocks all binding of anti-NOS1.
Figure S3. Systolic, diastolic and heart rates of flox and CDNOS1KO mice on low salt (LS) and six days of high salt (HS) diet. (A) CDNOS1KO have a significantly higher systolic blood pressured compared to flox control mice while on a HS diet. (B) On day 6 of HS diet, CDNOS1KO mice had a significantly higher diastolic pressure than flox mice. (C) There were no differences in heart rate between flox and CDNOS1KO mice, although both heart rate. (D) pulse pressure was also not different between the
genotypes of mice on LS or day 6 of HS. * P < 0.05 between flox and CDNOS1KO mice. † P < 0.05 LS compared to HS.

**Figure S4.** Food and water intake were similar between flox and CDNOS1KO mice on low salt (LS) and high salt (HS) diets. Water intake was significantly increased in both genotypes while on a HS diet (Genotype p = 0.94, Diet p < 0.001, interaction p = 0.90, N = 6)