Collecting Duct-Derived Endothelin Regulates Arterial Pressure and Na Excretion via Nitric Oxide

Markus P. Schneider, Yuqiang Ge, David M. Pollock, Jennifer S. Pollock, Donald E. Kohan

Abstract—Mice with a collecting duct-specific deletion of endothelin-1 are hypertensive and have impaired Na excretion. Because endothelin-1 activates NO synthase (NOS) in the collecting duct, we hypothesized that impaired renal NO production in knockout mice exacerbates the hypertensive state. Control and knockout mice were treated chronically with N\textsuperscript{G}-nitro-L-arginine methyl ester, and blood pressure (BP) and urinary nitrate/nitrite excretion were assessed. On a normal Na diet, knockout systolic BP was 18 mm Hg greater than in controls. N\textsuperscript{G}-nitro-L-arginine methyl ester increased BP in control mice by 30 mm Hg and 10 mm Hg in collecting duct-specific deletion of endothelin-1 knockout mice, thereby abolishing the difference in systolic BP between the groups. A high-Na diet increased BP similarly in both groups. Urinary nitrate/nitrite excretion was lower in knockout mice than in controls on normal or high Na intake. In separate experiments, renal perfusion pressure was adjusted in anesthetized mice, and urinary nitrate/nitrite and Na excretion were determined. Similar elevations of BP increased urinary Na and nitrate/nitrite excretion in control mice but to a significantly lesser extent in knockout mice. Isoform-specific NOS activity and expression were determined in renal inner medulla homogenates from control and knockout mice. NOS1 and NOS3 activities were lower in knockout than in control mice given normal or high-Na diets. However, NOS1 or NOS3 protein expressions were similar in both groups on normal or high-Na intake. These data demonstrate that collecting duct-derived endothelin-1 is important in the following: (1) chronic N\textsuperscript{G}-nitro-L-arginine methyl ester–induced hypertension; (2) full expression of pressure-dependent changes in sodium excretion; and (3) control of inner medullary NOS1 and NOS3 activity. (Hypertension. 2008;51:1605-1610.)

Key Words: ET-1 ■ NO ■ Na ■ natriuresis ■ diuresis ■ blood pressure

Collecting duct (CD)-derived endothelin-1 (ET-1) is an important regulator of blood pressure (BP) and renal Na excretion. ET-1 inhibits distal nephron Na/K ATPase and epithelial Na channel activity, whereas CD-specific knockout of ET-1 causes hypertension and impaired Na excretion. The natriuretic effect of ET-1 is attributable, at least partially, to activation of CD endothelin B (ET\textsubscript{B}) receptors, because CD-specific deletion of ET\textsubscript{B} receptors causes hypertension and reduced Na excretion. How ET-1 modulates renal Na excretion and BP is unknown; however, the NO pathway may be involved. ET-1 stimulates NO production in inner medullary CD via the ET\textsubscript{B} receptor and NO synthase (NOS) 1 (also known as nNOS). Inner medullary NOS1 and NOS3 (latter also known as eNOS) activity are reduced in rats with dysfunctional ET\textsubscript{B} receptors. In thick-ascending limbs, ET-1 enhances NO production through increased NOS3 activity with resultant inhibition of chloride transport. NO also inhibits Na transport in isolated cortical CD. Taken together, these studies suggest that NO is an important mediator of the natriuretic and antihypertensive effects of CD-derived ET-1.

One condition under which the CD ET-1/NO pathway may be of particular importance is increased Na excretion in response to elevations of renal perfusion pressure. Intrarenal NO generation and urinary excretion of NO metabolites are increased during pressure-dependent changes in sodium excretion, whereas NO inhibition attenuates the pressure-natriuretic response. Furthermore, although the proximal tubule plays a role in pressure natriuresis, the CD may also be involved.

Based on the above considerations, the current study tested the hypothesis that absence of CD-derived ET-1 increases NO activity. We examined urinary NO excretion and isoform-specific NOS activity in inner medullae of CD ET-1 knockout (KO) and control mice. To assess the functional consequences of CD-specific ET-1 KO on renal Na production, we examined urinary NO metabolite excretion and Na excretion during changes in renal perfusion pressure, as well as the effect of NOS inhibition on systemic BP.

Methods

Animals

All of the experiments were performed with approval from the institutional animal care and use committees at the Medical College.
of Georgia and the University of Utah. CD ET-1 KO mice and littermate control mice were generated as described previously. Mice were studied at 3 to 4 months of age and fed either a normal (0.44% NaCl) or a high-salt (4% NaCl) rodent diet, with free access to drinking water.

**Chronic BP and Nitrate/Nitrite Excretion Experiments**

A catheter was inserted into the right carotid artery, tunneled subcutaneously, and the attached radiotransmitter localized to the back. Continuous recording of arterial pressure and heart rate was performed by telemetry (Data Sciences International). Two days after the surgery, values were recorded for 2 days on a normal-salt diet. Subsequently, Nω-nitro-L-arginine methyl ester (L-NAME; 1 mg/mL, Alexis Biochemicals) was added to the drinking water, and BP and heart rate recorded for the next 3 days. Mice were then switched to a high-salt diet and drinking water containing L-NAME, and telemetry data were recorded for 5 days.

In separate experiments, mice were placed into Nalgene metabolic cages and acclimated for 3 days on a normal-salt diet. Mice were continued on the normal-salt diet, and urine was collected on the second day after the conclusion of the acclimation period. Mice were then switched to a high-salt diet, and urine was collected on the third day of the high-salt intake. Urine nitrate/nitrite (NOx) concentration was determined using Griess reagent and measuring fluorescence at 540 nm on a microplate reader.

**Pressure-Excretion Experiments**

After the induction of anesthesia, the right jugular vein was cannulated, and BSA (1%) and 0.75% fluorescein isothiocyanate–inulin (Sigma-Aldrich) were administered in saline at 0.4 µL/min per gram of body weight throughout the study. The left carotid artery was cannulated and connected to a pressure transducer for BP and heart rate measurements. The bladder was catheterized for urine collection. Ligatures were placed loosely around the celiac and the mesenteric arteries and around the infrarenal aorta. After 30 minutes of equilibration, baseline urine was collected for 30 minutes ("low" BP period). The ligatures around the celiac and mesenteric arteries were then tightened ("medium" BP period), and urine was collected for 30 minutes. Thereafter, the ligature around the aorta was closed ("high" BP period), and urine was collected for 30 minutes. The animals were euthanized, blood collected, and decapsulated kidney weight determined. For more details of the procedures, please see the data supplement available at http://hyper.ahajournals.org.

**Isoform-Specific NOS Activity and Expression**

Mice were fed normal- or high-salt diets for 7 days and inner medullary tissue was dissected, rapidly frozen, and homogenized as described previously. Protein concentrations were determined by the Bradford assay (Bio-Rad). NOS activity was assessed by the conversion of [3H]arginine to [3H]citrulline in the presence of optimal concentrations of cofactors, as described previously. Total NOS activity was determined using N-nitro-L-arginine (1 mmol/L). Nω-(1-imino-3-butenyl)-L-ornithine (1 µmol/L, Cayman Chemicals) was used to assess NOS1-specific activity, and 1400W-dihydrochloride (100 mmol/L; Cayman Chemicals) was used to assess NOS2-specific activity. NOS3-specific activity was calculated as total NOS activity (NOS1-specific activity + NOS2-specific activity). The inhibitory constants of Nω-(1-imino-3-butenyl)-L-ornithine for NOS1, NOS2, and NOS3 are 0.1, 60.0, and 12.0 µmol/L, respectively. The inhibitory constants of Nω-nitro-L-arginine methyl ester (L-NAME; 1 mg/mL, Alexis Biochemicals) were added to the drinking water, and BP and heart rate recorded for the next 3 days. Mice were then switched to a high-salt diet and drinking water containing L-NAME, and telemetry data were recorded for 5 days.

**Statistics**

All of the data are presented as means±SEs. The effect of mouse genotype on renal function during varying levels of BP, salt intake, or L-NAME administration was analyzed by 2-way ANOVA. Baseline parameters between genotypes were analyzed by unpaired Student t tests. The effect of mouse genotype and salt diet on NOS activity and expression was analyzed by 1-way ANOVA. Posthoc comparison of individual means was by Neuman-Keuls multiple comparison test. P<0.05 was considered statistically significant.

**Results**

**Chronic BP and NO Metabolite Excretion Experiments**

CD ET-1 KO mice had higher systolic BP than control mice (Figure 1). Heart rate was not different between the 2 groups of mice (data not shown). Administration of L-NAME increased systolic BP in both groups; however, the rise in systolic BP was greater in control than in CD ET-1 KO mice (Figure 1). After 2 days of L-NAME, systolic BP was similar in the 2 groups (Figure 1). The addition of a high-Na diet plus L-NAME increased systolic BP similarly in both groups (P<0.05 days 6 to 10 on high-Na diet+L-NAME versus days 4 to 5 of L-NAME alone; Figure 1). Mean and diastolic BP followed the same pattern as systolic BP, whereas pulse rate did not differ between the 2 groups after L-NAME treatment on a normal or high-Na diet (data not shown). Food and water intake were similar between CD ET-1 KO and control mice on a normal or L-NAME diet; L-NAME plus high-Na intake increased water intake in both groups but to a comparable degree (data not shown).

Urinary NOx excretion was lower in CD ET-1 KO mice than in controls under baseline conditions (measured on day 2 of a normal-Na diet; Figure 2). Administration of a high-Na
diet increased urinary NOx excretion in both groups, although it remained lower in CD ET-1 KO animals (measured on day 2 of a high-Na diet; Figure 2).

**Pressure-Excretion Experiments**

Flox control and CD ET-1 KO mice had similar body weights and sexes. There were no differences in hematocrit values measured at the end of the protocol (36.4±1.7% flox control versus 36.8±2.0% CD ET-1 KO). Total kidney weight (both kidneys) was similar between groups: 414±30 mg of flox control versus 419±33 mg of CD ET-1 KO.

Mean BP increased after tying the celiac and mesenteric arteries and increased further after tying off the abdominal aorta, with no differences between groups (Figure 3A). Heart rate decreased in both groups similarly (Figure 3B). Glomerular filtration rate (GFR) increased similarly in both groups during increases in renal perfusion pressure (Figure 3C). Urine flow rate also increased in parallel with pressure; however, urinary flow rate during the high-pressure period was greater in the flox controls than CD ET-1 KO mice (Figure 4A). Urinary Na excretion was blunted in CD ET-1 KO mice compared with flox controls (Figure 4B). During increases in renal perfusion pressure, the ET-1 excretion rate increased similarly in both groups (Figure 4C). NOx excretion increased in both groups, but the increase was less in flox controls (Figure 4D).

**Renal Inner Medullary NOS Activity and Expression**

During normal salt intake, total NOS activity in the inner medulla was lower in CD ET-1 KO mice versus flox controls (Figure 5A). Both NOS1 and NOS3 activities were blunted in homogenates from the CD ET-1 KO mice compared with flox controls. High-salt intake increased NOS activity in inner medulla from both groups (Figure 5A) when compared with mice on a normal-salt diet. Medullary NOS1 and NOS3 activity increased similarly in both groups, although activity in CD ET-1 KO mice remained lower than in flox controls (Figure 5B through 5D). In contrast to activity, NOS1 and NOS3 protein expression in the inner medulla were similar in flox control and CD ET-1 KO mice on a normal- or high-salt diet (please see the data supplement).

**Discussion**

Key findings of this study are as follows: (1) pressure-dependent changes in Na, water, and NO excretion are impaired in CD ET-1 KO mice; (2) renal NO production is reduced in CD ET-1 KO mice associated with decreased renal inner medullary NOS1 and NOS3 activity; (3) the difference in BP between CD ET-1 KO and control mice is abolished by NOS blockade; and (4) the hypertensive effect of L-NAME in
control mice is severely attenuated in CD ET-1 KO mice. These results indicate that CD-derived ET-1 regulates the pressure-induced changes in natriuresis and diuresis. CD-derived ET-1 effects are largely because of the activation of medullary NO. Finally, CD-derived ET-1–dependent NO production is a major factor in chronic NOS inhibition–induced hypertension.

Pressure-dependent changes in urinary Na excretion are partially dependent on renal NO production.11–15 Our findings indicate that CD-derived ET-1, likely through NO, modulates

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**Figure 4.** A, Urinary excretion volumes in flox control (n=7) and CD ET-1 KO mice (n=6) during periods of low, medium, and high BP. B, Urinary Na excretion in flox control mice and CD ET-1 KO mice during periods of low, medium, and high BP. C, Urinary ET-1 excretion in flox control and CD ET-1 KO mice during periods of low, medium, and high BP. D, Urinary NOx excretion in flox control and CD ET-1 KO mice during periods of low, medium, and high BP.

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**Figure 5.** A, Isoform-specific NOS activity in renal inner medullary homogenates from flox control (n=8) and CD ET-1 KO mice (n=8) during normal-salt (NS) and high-salt (HS) diets. *P<0.05 vs flox control on the same diet; †P<0.05 vs normal-Na diet in same mouse genotype.
the pressure-natriuresis relationship. Because ET-1 acts in an autocrine/paracrine fashion, the absence of CD-derived ET-1 presumably decreases local NO generation during pressure natriuresis. Potential sites of ET-1-regulated NO generation are the CD or neighboring cells, such as interstitial or endothelial cells. Nephron segments proximal to the CD also participate in pressure-natriuresis; the involvement of ET-1 and NO in Na reabsorption in these regions needs investigation.

We observed increased renal ET-1 excretion (which derives entirely from the kidney) with increased renal perfusion pressure, suggesting that production and/or secretion of renal ET-1 is acutely modified by perfusion pressure. During the “low-pressure” period, urinary ET-1 excretion was less in the CD ET-1 KO mice, consistent with previous findings. Urinary ET-1 excretion rose to comparable levels between the 2 groups when renal perfusion pressure increased, suggesting that urinary ET-1 excretion, under these conditions, reflects ET-1 production by sites other than the CD. However, the vast majority of ET-1 is secreted abluminally; hence, urinary ET-1 excretion is not a likely sensitive marker of CD-derived ET-1 release. How perfusion pressure increases CD ET-1 production is speculative, although one possibility is tubule flow rate; shear stress increases endothelial cell ET-1 production.

Two other aspects of the pressure-excretion studies deserve comment. First, the BP difference between CD ET-1 KO and control mice was abolished by isoflurane anesthesia. It is conceivable that the substantially lower BP during anesthesia obscures differences found in conscious animals. Importantly, during the induction of natriuresis by increased renal perfusion pressure, BP rose similarly in the 2 groups, allowing the comparison of changes in renal parameters. Second, GFR values during the low-pressure period were lower than previously reported for anesthetized mice, possibly because of BP being at the lower end of the renal blood flow autoregulation range. This could also explain why GFR was not tightly autoregulated when BP was increased during pressure natriuresis. However, GFR was similar between CD ET-1 KO and control mice throughout the pressure range. Two major findings were observed during l-NAME administration. First, the BP difference between the 2 groups of mice was abolished by l-NAME, strongly implicating NO as an effector of CD-derived ET-1 actions. The reduction in urinary NOx excretion and NOS activity in CD ET-1 KO mice supports this conclusion. Second, l-NAME markedly increased BP in controls, but only modestly elevated BP in CD ET-1 KO mice. It is remarkable that NO, which depends on CD-derived ET-1, presumably from the CD and adjacent cells, greatly contributed to l-NAME–induced hypertension. This finding underscores the importance of the CD ET-1/NO pathway in the control of systemic BP. Notably, another ET-1–regulated natriuretic factor, prostaglandin E2, is not involved in the hypertensive phenotype of CD ET-1 KO mice. ET-1 stimulates CD prostaglandin E2 production; however, blockade of cyclooxygenase does not alter BP in these mice.

The finding that that CD-derived ET provides a tonic stimulatory signal to both NOS1 and NOS3 in the renal inner medulla agrees with previous studies. Rats deficient in ET\(_B\) receptors have reduced inner medullary NOS1 and NOS3 activities. ET-1 induces NO release in CD cells via activation of NOS1, whereas ET-1 increases expression of NOS3 in CD cells. High Na intake increases medullary NOS activity and ET-1 production. High Na intake increased NOS1 and NOS3 activities in the inner medulla in both mouse genotypes similarly, suggesting that the high-salt diet–induced NOS activity may not be via CD-derived ET. Yet, NOS activity from the CD ET-1 KO mice on a high-salt diet was lower than in control mice. NOS1 and NOS3 expression in the medullary homogenates were similar in all of the groups; thus, the decreased activity may be mediated by posttranslational modification of the NOS isoforms. The cellular sources of the NOS isoforms were not determined in the current study; clearly CD, endothelial, and interstitial cell sources are possibilities.

Although these studies provide support for the CD endothelin/NO system in the control of excretory function, there remains the question of hemodynamic versus tubular actions of ET\(_B\) receptor-dependent NO production. Several laboratories have shown that ET\(_B\) receptor activation increases blood flow within the renal medulla, which could facilitate improved Na excretion. We have reported that a high-salt diet increases ET\(_B\) receptor expression and vasodilator activity within the preglomerular vessels of juxtamedullary nephrons that feed the medullary circulation. The balance between hemodynamic and direct tubular actions will require further study.

Perspectives
This study highlights the physiological importance of the CD ET-1/NO axis in regulating BP. Our experiments demonstrate that pressure-dependent changes in Na and water excretion require CD-derived ET-1 activation of the NO pathway. This most likely involves activation of NOS1 and/or NOS3. These findings are particularly important in the context of hypertension, where there is an altered relationship between BP and Na excretion. It is possible that defects in this pathway could account for elevations in BP in hypertension because of a variety of potential mechanisms, including reduced CD-dependent ET-1 synthesis, reduced NOS expression and/or activity, and others.

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Disclosures
None.
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Collecting duct-derived endothelin regulates arterial pressure and Na excretion via nitric oxide

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METHODS

*Pressure-excretion experiments*

Mice were anesthetized with isoflurane at a concentration between 1.0 to 1.5% isoflurane in 95% O\textsubscript{2} as required to achieve adequate depth of anesthesia. Body temperature was measured with a rectal probe and kept stable around 37.5 °C using a servo-controlled surgical heating table. After the induction of anesthesia, the right jugular vein was accessed with a polyethylene catheter size 10 (PE 10). Bovine serum albumin (1%) and 0.75% fluorescein isothiocyanate (FITC)-inulin (Sigma-Aldrich, St. Louis, MO) were administered in saline at a rate of 0.4 µl/min/g body weight continuously throughout the study. The left carotid artery was cannulated (PE 10) and connected to a pressure transducer for direct BP and heart rate measurements. Hemodynamic data were recorded and analyzed with a Powerlab data acquisition system (ADInstruments, Boston, MA).

After an abdominal incision, the bladder was catheterized for urine collections (PE 50). The venous catheter, the bladder catheter and the urine collection tube were all wrapped in tin foil to prevent FITC exposure to light. Ligatures were placed loosely around the celiac and the mesenteric arteries, and around the abdominal aorta below the renal arteries. During the abdominal surgery, an additional heating lamp was used to prevent heat loss from exposure of the intestines, and animals were covered with gauze after completion of surgery.
After a 30-minute equilibration period, urine was collected for 30 minutes to determine baseline renal function ("low" BP period). After the 30-minute baseline period, the ligatures around the celiac and mesenteric arteries were tightened to permit an increase of BP ("medium" BP period) and urine was collected during the following 30-minute period. Thereafter, the ligature around the aorta was closed to permit a further, maximal increase in BP ("high" BP period) and urine was collected during this final 30-minute period. The animals were sacrificed with a high dose of isoflurane, blood was collected by cardiac puncture and kidneys removed, decapsulated and weighed. Urine volumes obtained during each collection period were determined gravimetrically. Plasma and urine samples were snap-frozen in liquid nitrogen until further analysis.

**Isoform-specific NOS activity and expression**

For NOS activity, homogenates were incubated with [³H]arginine (10 µM final arginine, 71 Ci/mmol; Amersham, Arlington Heights, IL) in the presence of 1 mM NADPH, 30 nM calmodulin, 3 µM tetrahydrobiopterin, 2 mM CaCl₂, 1 µM FAD, and 1 µM FMN in a final volume of 50 µl for 30 minutes at room temperature.

For NOS isoform expression, following transfer of protein onto PVDF, membranes were blocked in 5% milk in Tris-buffered saline. Two-color immunoblots were performed using primary antibodies to NOS1 (1:1000; Santa Cruz; Santa Cruz, CA), NOS3 (1:250; BD Transduction; Franklin Lakes, NJ) in conjunction with a monoclonal antibody to actin (1:5000; Sigma, St. Louis, MO).
Specific bands were detected using the Odyssey Infrared Imager (LI-COR Biosciences, Lincoln, NE); AlexaFluor 680 was used for the detection of anti-NOS antibody (Molecular Probes, Eugene, OR) and IRDye800 (Rockland, Gilbertsville, PA) was used for the detection of anti-actin antibody. All densitometric results for NOS1 and NOS3 are reported normalized to actin densitometry. Densitometry was performed using a digital imaging system (Odyssey Infrared Imaging System, version 1.2; LI-COR Biosciences, Lincoln, NE).
A. NOS1 expression

Relative densitometry (NOS1:actin)

FLOX | CD ET-1 KO

KO | NS HS | NS HS | NS HS | NS HS

NOS1

actin

B. NOS3 expression

Relative densitometry (NOS3:actin)

FLOX | CD ET-1 KO

KO | NS HS | NS HS | NS HS | NS HS

NOS3

actin
Figure S1: NOS1 (Panel A) and NOS3 (Panel B) expression in renal inner medullary homogenates from Flox control (n=8) and CD ET-1 KO mice (n=8) during normal salt (NS) and high salt (HS) diets. Representative blots are presented for both NOS1 and NOS3 along with corresponding blots for actin. There were no significant differences between strain or salt diet groups.