Renal medullary ET<sub>B</sub> receptors produce diuresis and natriuresis via NOS1

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Nakano DS, Pollock JS, Pollock DM. Renal medullary ET<sub>B</sub> receptors produce diuresis and natriuresis via NOS1. Am J Physiol Renal Physiol 294: F1205–F1211, 2008. First published February 27, 2008; doi:10.1152/ajprenal.00578.2007.—Endothelin-1 (ET-1) plays an important role in the regulation of salt and water excretion. Infusion of the ET<sub>B</sub> receptor agonist sarafotoxin S6c (S6c: 0.45 μg·kg<sup>–1</sup>·h<sup>–1</sup>) in the renal medulla of anesthetized, male Sprague-Dawley rats markedly increased the urine flow (UV) and urinary sodium excretion (UNaV) by 67 and 120%, respectively. This was associated with an increase in medullary cGMP content but did not affect blood pressure. In addition, S6c-induced diuretic and natriuretic responses were absent in ET<sub>B</sub> receptor-deficient rats. Confusion of N<sup>ω</sup>-propyl-l-arginine (10 μg·kg<sup>–1</sup>·h<sup>–1</sup>) or Rp-8-Br-PET-cGMPs (10 μg·kg<sup>–1</sup>·h<sup>–1</sup>) or RQIKWFiQRRMWWK<sub>KK</sub>-LRK<sub>5</sub>H-amide (18 μg·kg<sup>–1</sup>·h<sup>–1</sup>) or PKG inhibitor, also inhibited S6c-induced increases in UV and UNaV. These results demonstrate that renal medullary ET<sub>B</sub> receptor activation induces diuretic and natriuretic responses through a NOS1, cGMP, and PKG pathway.

sodium excretion; nitric oxide synthase 1; guanosine 3′,5′-cyclic monophosphate; protein kinase G

ENDOTHELIN-1 (ET-1), originally described as being released from vascular endothelial cells (46), is now known to be produced by many cell types and exerts a variety of biological effects in various organ systems (27). The renal medulla is a major site of ET-1 synthesis in the body (14, 42). Considerable evidence has accumulated in recent years to demonstrate the physiological importance of renal medullary ET-1 in the control of arterial pressure and salt sensitivity. In inner medullary collecting duct (IMCD) cells, ET-1 reduced ouabain-sensitive sodium excretion; nitric oxide synthase 1; guanosine 3′,5′-cyclic monophosphate; protein kinase G.

METHODS

Materials

S6c was obtained from American Peptide (Sunnyvale, CA). N<sup>ω</sup>-propyl-l-arginine (NPA), Rp-8-Br-PET-cGMPs (Rp-cGMP), and BSA were purchased from Calbiochem (La Jolla, CA). PKG inhibitor peptide RQIKWFiQRRMWWK<sub>KK</sub>-LRK<sub>5</sub>H-amide (DT-3) was purchased from Axxora (Bingham, UK). All other reagents were obtained from Sigma Chemical (St. Louis, MO).

Animals

Experiments used male Sprague-Dawley (SD) rats (250–350 g) from Harlan Laboratories (Indianapolis, IN). Additional experiments used male wild-type (control) and homozygous (slsl) rats deficient of ET<sub>B</sub> receptors (Wistar genetic background) obtained from our local breeding colony. Both control and slsl rats carry the transgene for dopamine-β-hydroxylase that rescues the ET<sub>B</sub>-deficient rats from a specific expression of ET-1 within the renal collecting duct develop hypertension that is salt dependent (1).

Nitric oxide (NO) is thought to act as the downstream mediator of renal ET<sub>B</sub> receptor activation, because total NO synthase (NOS) inhibition can inhibit ET<sub>B</sub> receptor-dependent responses both in vivo (17) and in vitro (22). However, which NOS isoform is mainly involved in ET<sub>B</sub>-mediated diuretic and natriuretic responses remains unclear because all three NOS isoforms are expressed in tubules within the renal medulla (18, 33). Previous studies have demonstrated that NOS1 contributes to roughly 40% of basal NO production in the renal medulla (12). Inhibition of renal medullary NOS1 by intrarenal infusion of anti-sense oligonucleotides or specific enzyme inhibitors elevated the blood pressure in rats fed a high-salt diet (19), thus indicating the importance of medullary NOS1 on control of sodium balance. Moreover, Stricklett et al. (35) have shown that ET-1 stimulated both NO and cGMP production in rat IMCD cells and that an ET<sub>B</sub> receptor antagonist or NOS1 selective inhibitor suppressed this increase in NO production. In addition, feeding rats a high-salt diet, which is known to increase renal ET-1 production (29), has been reported to increase the expression of NOS1 protein in the IMCD (26), although this has not been a consistent finding (36). Taken together, these observations led us to hypothesize that ET<sub>B</sub> receptors within the renal medulla produce a diuretic and natriuretic effect by activation of NOS1 as a means of facilitating salt excretion in vivo. Thus we determined the effect of renal medullary infusion of an ET<sub>B</sub> receptor selective agonist, sarafotoxin 6c (S6c), on urinary sodium and water excretion in normotensive rats. Furthermore, we examined whether NOS1, cGMP, and/or protein kinase G (PKG) could be effectors of the downstream signaling events during ET<sub>B</sub> receptor-dependent responses.
lethal phenotype by expressing a functional ETB receptor in adrenergic tissues (7, 37). The Medical College of Georgia Institutional Animal Care and Use Committee approved experimental protocols and animal care procedures in all experiments.

Surgical Preparation

Rats were anesthetized with inactin (100 mg/kg ip) and placed on a servo-controlled heating table to maintain rectal temperature constant at 37°C. The trachea was cannulated (PE-205) to facilitate respiration. The right femoral artery was catheterized for monitoring blood pressure using a MacLab data acquisition system (AD Instruments, Milford, MA), and the right jugular vein was catheterized for infusion of PBS (0.9% NaCl) containing 6.2% BSA at a rate of 1.8 ml/h to maintain euvolemia. A midline incision was made, and a stretched PE-10 catheter was inserted 5 mm in the left kidney and secured to the renal capsule. After the catheter was inserted, saline (0.9% NaCl) was infused directly in the renal medulla at 0.5 ml/h. Medullary blood flow was measured by single-fiber, laser Doppler flowmetry as previously described (39). Urine was collected separately from the left and right kidneys via catheters placed in each ureter. Experiments were started after an 80-min equilibration period. At the end of each experiment, the kidneys were dissected to ensure the catheter was in the appropriate position within the medulla.

Protocol 1. After two 20-min control urine collection periods, increasing doses of S6c (0.15, 0.45 and 1.5 µg·kg⁻¹·h⁻¹) were infused in the renal medullary interstitium of SD rats for two 20-min experimental periods at each dose. A separate group of rats received an infusion of saline vehicle during control and experimental periods.

Protocol 2. Again, following two 20-min control periods, increasing doses of S6c (0.15 and 0.45 µg·kg⁻¹·h⁻¹) were infused in the renal medullary interstitium of ETB-deficient or control rats for two 20-min experimental periods at each dose.

Protocol 3. Following control periods, increasing doses of S6c (0.15 and 0.45 µg·kg⁻¹·h⁻¹) were infused in the renal medullary interstitium of SD rats. In additional groups of rats, the NOS1

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**Fig. 1.** Urine flow (top), sodium excretion (middle), and mean arterial pressure (bottom) during medullary interstitial infusion of sarafotoxin S6C (S6c) in the left kidney. Data are expressed as means ± SE; n = 4–5 rats/group. *P < 0.05 and **P < 0.01 vs. saline group.

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**Fig. 2.** Renal medullary infusion of S6c in the left kidney increased the urine flow greater in wild-type rats compared with that in endothelin (ET) type B (ETB)-deficient rats. Effect of intramedullary infusion of S6c on urine flow (top), sodium excretion (middle), and mean arterial pressure (bottom) in ETB-deficient rats or in wild-type rats. Data are expressed as means ± SE; n = 3–6 rats/group. *P < 0.05 vs. ETB-deficient group.
After NPA infusion, the drug was changed to methyl ester (L-NAME; 100 g/kg) for two 20-min experimental periods at each dose. Measurement of medullary cGMP content was increased when the highest dose of 1.50 μg·kg⁻¹·h⁻¹ of S6c was infused in the left renal medulla, possibly through a renorenal reflex or spillover from the medullary infusion in the systemic circulation. Because of this effect in contralateral kidney, we used lower doses (0.15 and 0.45 μg·kg⁻¹·min⁻¹) in the following experiments. Mean arterial pressure was not significantly affected by S6c infusion.

To further assess the specific role of ETB receptors in the excretory response to intramedullary infusion of S6c, we conducted similar experiments in ETB-deficient rats and their genetic controls (Fig. 2). Medullary infusion of S6c induced a significant diuretic and natriuretic response in control rats that was not evident in ETB-deficient rats.

To estimate the involvement of NOS1 on ETB receptor-dependent diuretic and natriuretic responses, we determined the effects of treatment with NPA, a selective NOS1 inhibitor, on the response to S6c (Fig. 3). Intramedullary infusion of NPA markedly suppressed the S6c-induced diuretic and natriuretic responses. Infusion of NPA without S6c had no effect on sodium or water excretion at the dose that inhibited the S6c

**RESULTS**

Renal medullary infusion of S6c dose-dependently increased both the urine flow and sodium excretion from the left kidney compared with infusion of saline (Fig. 1). Urine flow and sodium excretion from the right kidney was increased when the left renal medulla was infused, possibly through a renorenal reflex or spillover from the medullary infusion in the systemic circulation. Because of this effect in contralateral kidney, we used lower doses (0.15 and 0.45 μg·kg⁻¹·min⁻¹) in the following experiments. Mean arterial pressure was not significantly affected by S6c infusion.

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**Measurement of Medullary cGMP Content**

cGMP was extracted from the tissue according to the protocol of Brophy et al. (3). The medullas were homogenized in 500 μl of ice-cold 10% TCA using a glass-Teflon homogenizer. The homogenate was centrifuged 15,000 g for 15 min at 4°C. The supernatant was washed four times with 5 vol of water-saturated ether, and the upper phase was discarded with each wash. The aqueous extract was dried using a vacuum evaporator (Jouan, Winchester, VA). The dried extract was reconstituted in 125 μl of acetate buffer (50 mM, pH 6.2) and was saved for measurement of cGMP content by RIA (30). The pellet was resuspended in 500 μl of 1 N NaOH, and protein concentrations were determined by standard Bradford assay (Bio-Rad Laboratories, Hercules, CA) using BSA as the standard.

**Statistical Analysis**

All values were expressed as means ± SE. For statistical analysis, we used one-way ANOVA followed by Tukey-Kramer multiple-comparison tests. Differences were considered significant at P < 0.05.

![Graph showing effect of selective nitric oxide synthase (NOS) 1 inhibitor N^G- propyl-L-arginine (NPA) on the response to intramedullary S6c infusion in the left kidney: urine flow (top), sodium excretion (middle), and mean arterial pressure (bottom). Data are expressed as means ± SE; n = 7–8 rats/group. *P < 0.05 and **P < 0.01 vs. saline group. †P < 0.01 vs. S6c group.](image)

![Graph showing cGMP content in renal medullary tissue following intrarenal medullary infusion of saline, S6c, or S6c plus the NOS1 inhibitor NPA in the left kidney. Data are expressed as means ± SE; n = 4–5 rats/group. **P < 0.01 vs. saline group. ††P < 0.01 vs. S6c group.](image)
response. No significant change was observed in urine flow or sodium excretion from the right kidney in any group. Likewise, mean arterial pressure was not changed in any group.

Because cGMP is a well-established second messenger of NO, we determined renal medullary cGMP content in animals given intramedullary infusion of saline, S6c, and S6c plus NPA (Fig. 4). The left renal medulla of the S6c-infused group had a significantly greater amount of cGMP compared with the saline-infused group. NPA treatment completely suppressed the S6c-induced increase in cGMP content in renal medulla.

We further estimated the involvement of cGMP on S6c-induced diuretic and natriuretic responses by using the PKG inhibitor Rp-cGMP or DT-3 (Fig. 5). Coinfusion of PKG inhibitor with S6c significantly inhibited the S6c-induced increase in urine flow and sodium excretion from the left kidney. The infusion of Rp-cGMP alone, but not DT-3, slightly but significantly increased the sodium excretion during 80–120 min after the start of the infusion (data not shown). Urine flow rate and sodium excretion from the right kidney were unaffected by each PKG inhibitor infusion in the left kidney. No significant changes in mean arterial pressure were observed in any group.

Although NPA has high selectivity for the NOS1 in vitro, the selectivity in vivo has not been clarified yet. Therefore, to estimate the selectivity of NPA in vivo, we measured the changes in renal medullary blood flow in response to NPA or l-NAME, a nonselective NOS inhibitor (Fig. 6). The medullary blood flow was unaffected by infusion of NPA, even at 100 μg·kg⁻¹·h⁻¹; a dose 10 times higher than necessary to inhibit the S6c-induced natriuresis. Subsequent infusion of l-NAME significantly decreased the medullary blood flow.

**DISCUSSION**

The evidence that ET-1 functions as a physiological regulator of renal sodium and water excretion has been slowly accumulating since the first identification of intrarenal ET-1 production along with a high concentration of ET₁ receptors...
within the renal medulla (24). Our studies demonstrate that direct intramedullary stimulation of ETB receptors results in a diuretic and natriuretic response, which agrees with considerable in vitro data that ETB receptor stimulation inhibits renal tubular reabsorption (6, 22, 38). Furthermore, we report that renal ETB-dependent increases in sodium and water excretion depend on the activity of NOS1 and appear to also function via cGMP stimulation of PKG.

Early studies examining the effect of systemically administered ET-1 did not agree on whether ET-1 increased or decreased urine flow and sodium excretion due to increases in arterial pressure or decreases in the filtered load of sodium (32). Our studies employed the interstitial infusion technique (19) to minimize effects of ET receptor activation on renal perfusion pressure or glomerular filtration rate. Because we did not observe any changes in mean arterial pressure during intramedullary infusion of S6c, we can eliminate the possibility that the increase in urine sodium and water excretion was due to changes in renal perfusion pressure.

We also used the highly selective ETB agonist S6c to focus on ETB receptor-specific effects (43). ETB receptors appear to be the predominant receptor subtype that accounts for the ability of ET-1 to inhibit sodium reabsorption (6, 22). It has been established for many years that ETB receptors in the vascular endothelium stimulate NO production (11, 40). Furthermore, the ability of the ETB receptor to reduce renal tubular transport has been attributed to increasing NO (22). Matsuo and colleagues (17) have also reported that S6c infusion in the renal artery of anesthetized dogs results in a natriuretic and diuretic response, which agrees with consideration of PKG.

A number of studies have shown that the ETB receptor system can produce vasodilation within the renal microcirculation, and in particular, within the renal medulla (9, 31, 39). Although increasing blood flow within the renal medullary circulation may contribute to reduced renal salt and water reabsorption, one could assume that ETB-mediated vasodilation would utilize the endothelial isoform, NOS3, and not NOS1, since ETB-mediated vasodilation is endothelial-dependent and there have been no reports of NOS1 expression in endothelial cells. Kakoki et al. (12) have shown that intravenous infusion of a selective NO inhibitor does not influence medullary blood flow despite the fact that medullary NO levels were significantly decreased. This evidence is consistent with our result that shows intramedullary infusion of L-NAME but not NPA decreases the medullary blood flow. Again, this observation suggests that the ETB/NOS1 pathway is not through a hemodynamic mechanism. Therefore, our current findings of NOS1 dependency in the natriuretic response to S6c would suggest that a hemodynamic mechanism is unlikely and that the NOS1-dependent response to S6c is primarily due to an inhibitory effect on the tubular sodium reabsorption. Nonetheless, there remains the possibility that renal tubular NO production could contribute to hemodynamic effects within the renal medulla as suggested by Cowley and colleagues (4).

An additional new finding in the present study is that the selective NOS 1 inhibitor NPA not only inhibited the diuretic and natriuretic actions of S6c but also inhibited S6c-induced increases in renal medullary cGMP content. These data agree with the hypothesis that ETB-dependent NO production inhibits renal tubular reabsorption through NOS1-dependent generation of NO and subsequent cGMP generation. Furthermore, participation of PKG appears likely, since the competitive inhibitor Rp-cGMP or DT-3 also inhibited the renal excretory response to S6c. cGMP has been known for many years to be an important factor in regulating renal tubular sodium reabsorption, especially in terms of the activity of factors such as atrial natriuretic peptide and guanylin (34, 48).

A potential limitation to the present study is the selectivity of the pharmacological inhibitors in vivo. The selectivity of NPA for NOS1 is 150- and 3,200-fold greater than that of NOS3 and NOS2 in vitro (49), but there have been no studies to clearly demonstrate selectivity of NPA for the NOS1 in vivo. We observed that doses of NPA that blocked the response to S6c (10 and 100 μg·kg⁻¹·min⁻¹) had no effect on blood flow in the renal medulla (Fig. 6). However, a dose of the nonselective NOS inhibitor L-NAME (100 μg·kg⁻¹·min⁻¹) produced a profound decrease in medullary blood flow consistent with inhibition of NOS3. The result indicates that NPA infusion...
does not appear to inhibit NOS3. In addition with this experiment, we attempted to confirm the involvement of NOS1 by using other NOS inhibitors, such as N⁵-(1-imino-3-butenyl)-ornithine or 7-nitroindazole. However, intramedullary infusion of these agents themselves induced strong diuretic responses (unpublished observations). In addition, even NPA increased the urine flow when we used the dosage higher than 100 \( \mu g \cdot kg^{-1} \cdot h^{-1} \). The reasons for this apparent contradictory action of NOS1 revealed by administration of the other inhibitors and the higher dose of NPA is not known but may be due to spillover to inhibit cortical NOS1 in the macula densa to reduce tubuloglomerular feedback or effects on nerve activity. NOS1 was originally named neuronal NOS because it was first discovered in neuronal tissue and is known to play a critical role in modulating sympathetic nerve activity.

Mattson and Bellemure (19) observed that chronic infusion of NOS1 antisense oligonucleotides or the NOS1-specific inhibitor 7-nitroindazole resulted in chronic hypertension. More recently, Mattson and Meister (20) observed that NOS3 does not account for salt sensitivity in mice treated with the non-specific NOS inhibitor L-NAME. These findings are consistent with an important role for one of the other NOS isoforms, such as NOS1, in determining salt sensitivity. The current study provides additional evidence to link the ETB receptor and NOS1 activity within the renal medulla in the control of sodium excretion. A seemingly contrasting observation to this overall hypothesis is that NOS1 knockout mice have normal blood pressures (21). However, these mice only lack the NOS1c and may express other splice variants that could compensate. Furthermore, the NOS1c knockout mice have not been characterized in terms of their ability to excrete salt and water.

Considerable evidence has demonstrated an important role for ETB receptors in the regulation of blood pressure control of renal sodium excretion. Our laboratory has previously shown that chronic blockade of ETB receptors results in salt-dependent hypertension (1). Likewise, Gariepy et al. (7) have shown that rats with a genetic mutation of the ET-1 receptor causes hypertension and sodium retention. In any event, our studies demonstrate an important link between ETB-dependent changes in sodium and water excretion in the renal medulla and the activity of NOS1, cGMP, and PKG in a normotensive rat.

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