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

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Indeed, rats or mice lacking functional ETB receptors display an important role in the regulation of salt and water excretion. Effects of ETB receptor stimulation in vivo. Infusion of the ETB 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 ETB receptor-deficient rats. Confusion of N<sup>3</sup>-propyl-L-arginine (10 μg·kg<sup>-1</sup>·h<sup>-1</sup>), a selective NOS1 inhibitor, suppressed S6c-induced increases in UV, UNaV, and medullary cGMP concentrations. Rp-8-Br-PET-cGMPS (10 μg·kg<sup>-1</sup>·h<sup>-1</sup>) or RQIKIWFQNRRMKWKK-LRK<sub>H</sub>-amide (18 μg·kg<sup>-1</sup>·h<sup>-1</sup>), a PKG inhibitor, also inhibited S6c-induced increases in UV and UNaV. These results demonstrate that renal medullary ETB receptor activation induces diuretic and natriuretic responses through a NOS1, cGMP, and PKG pathway.

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

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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

![Graph](http://ajprenal.physiology.org/)

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.

![Graph](http://ajprenal.physiology.org/)

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.
stored at ately frozen in liquid nitrogen for cGMP measurement. Samples were
g/H9262 After NPA infusion, the drug was changed to
tium of SD rats for two 20-min experimental periods at each dose.
methyl ester (L-NAME; 100 μg/kg) was infused in the renal medullary intersti-
Fig. 3. Effect of the selective nitric oxide synthase (NOS) 1 inhibitor N
d-propyl-L-arginine (NPA) on the response to intramedullary S6c infusion in the
left kidney (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.

inhibitor NPA (10 μg·kg⁻¹·h⁻¹) (49), PKG inhibitor Rp-cGMP (10 μg·kg⁻¹·h⁻¹) (41), or DT-3 (18 μg·kg⁻¹·h⁻¹) (5) was infused in
the renal medulla beginning 40 min before S6c and was continued
during S6c infusion.

Protocol 4. Following control periods, increasing doses of NPA (10 and 100 μg·kg⁻¹·h⁻¹) were infused in the renal medullary interstiti-
num of SD rats for two 20-min experimental periods at each dose.
After NPA infusion, the drug was changed to N⁴-nitro-l-arginine methyl ester (l-NAME; 100 μg·kg⁻¹·h⁻¹).

The left renal medulla was dissected at the end of the infusion
periods in the saline, S6c, and S6c + NPA groups and was immedi-
ately frozen in liquid nitrogen for cGMP measurement. Samples were
stored at −80°C until analysis.

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 homoge-
nate 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 concentra-
tions were determined by standard Bradford assay (Bio-Rad Lab-
oratories, 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.

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
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 circula-
tion. 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 af-
fected by S6c infusion.

To further assess the specific role of ETB receptors in the
excretory response to intramedullary infusion of S6c, we con-
ducted 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 natri-
uretic responses. Infusion of NPA without S6c had no effect on
sodium or water excretion at the dose that inhibited the S6c

Fig. 4. 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.
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 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 ET₄ receptors

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**Fig. 5.** Effect of the protein kinase G (PKG) inhibitor Rp-8-Br-PET-cGMPS (Rp-cGMP) or RQIKIWFQNRRMKWKK-LRK-amide (DT-3) 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 = 5–7 rats/group. *P < 0.05 and **P < 0.01 vs. saline group, †P < 0.05 vs. S6c group.
proexcretory effects of ETB receptor stimulation. Herrera and colleagues (17) have also reported that S6c infusion in 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 evidence in vitro 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.

Fig. 6. Change in medullary blood flow in responses to intramedullary infusion of NPA and an NOS1 inhibitor L-NAME. Data are normalized by the flow at the first 20 min and are expressed as means ± SE; n = 4 rats.

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 effect that is also associated with increases in urinary excretion of NO metabolites, nitrate and nitrite. A lingering question has been which NOS isofrom could be involved in the proexcretory effects of ETB receptor stimulation. Herrera and Garvin (10) have shown that ETB receptor stimulation is responsible for increases in NOS3 expression in the thick ascending limb. This same laboratory has shown that NO-dependent inhibition of chloride reabsorption involves NOS3 and not NOS1 (23). In cultured collecting duct cells, however, Stricklett et al. (35) showed that ET-1 increases NO production via NOS1 activation, but not NOS2 or NOS3. Although all three NOS isoforms are expressed in both the thick ascending limb and the collecting duct, microdissection experiments suggest that the greatest amount of constitutive NOS activity is within the medullary collecting duct system (45), the same location as the greatest amount of ET-1 synthesis and ETB receptor expression (13, 15). Therefore, it is tempting to speculate that the natriuretic and diuretic response to S6c observed in our experiments using intramedullary infusion is related to activity within the collecting duct although this idea awaits further clarification.

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 NOS1 inhibitors, such as Nω-(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 μg·kg⁻¹·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 Bellehumeur (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 NOS1α and may express other splice variants that could compensate. Furthermore, the NOS1α 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 via control of renal sodium excretion. Our laboratory has previously shown that chronic blockade of ETB receptors results in hypertension that is salt dependent (44). Likewise, Gariepy et al. (7) have shown that rats with a genetic mutation of the ETB receptor also display salt-dependent hypertension. A more specific role for ET-1 and ETB receptors within the collecting duct is derived from recent studies demonstrating that the tissue-specific deletion of ET-1 or ETB receptors from the collecting duct results in salt-dependent hypertension (1, 8). The current study extends these findings although the precise mechanisms by which ETB receptors transmit signals to increase urine sodium and water excretion through NOS1, cGMP, and PKG will need to be elucidated in future studies.

**Perspectives**

A role for ET-1 and the renal ETB receptor in the long-term control of renal salt and water excretion now appears well established given results from genetic deletion studies as well as pharmacological experiments (1, 7, 8, 25). Improvements in our understanding of the downstream signaling pathways for the renal ETB receptor will provide further insight into the specific role of the ET-1/ETB system in controlling excretory function. It is expected that the relationship among ETB, NOS, and urine excretion may change along with the development of renal-dependent disorders, such as salt-sensitive hypertension. We speculate that factors interfering with the ET-1/ETB signaling pathway could contribute to salt-induced elevations in blood pressure. In any event, our studies demonstrate an important link between ET1-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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