Renal Medullary Endothelin-1 is Decreased in Dahl Salt Sensitive Rats

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Abstract

Although it is well established that the renal endothelin (ET-1) system plays an important role in regulating sodium excretion and blood pressure through activation of renal medullary ET\textsubscript{B} receptors, the role of this system in Dahl salt sensitive (DS) hypertension is unclear. The purpose of this study was to determine if the DS rat has abnormalities in the renal medullary endothelin system when maintained on a high sodium intake. The data indicates that Dahl salt resistant rats (DR) on a high salt diet had a 6-fold higher urinary endothelin excretion than in the DR rats on a low Na\textsuperscript{+} intake (17.8±4 pg/day versus 112±44 pg/day). In sharp contrast, urinary endothelin levels increased only 2 fold in DS rats in response to a high Na\textsuperscript{+} intake (13±2 pg/day vs. 29.8±5.5 pg/day). Medullary endothelin concentration in DS rats on a high Na\textsuperscript{+} diet was also significantly lower than DR rats on a high Na\textsuperscript{+} intake (31 ± 2.8 pg/mg versus 70.9±5 pg/mg). Furthermore, DS rats had a significant reduction in medullary ET\textsubscript{B} receptor expression as compared to DR rats while on a high Na\textsuperscript{+} intake. Finally, chronic infusion of ET-1 directly into the renal medulla blunted Dahl salt-sensitive hypertension. These data indicate that a decrease in medullary production of ET-1 in the DS rat could play an important role in the development of salt sensitive hypertension observed in the DS rat.
**Introduction**

Endothelin-1 (ET-1) was first characterized in 1988 as a potent vasoconstrictor released by endothelial cells (26). Two receptor subtypes have been since identified, ETA and ETB (24). Both of these receptors are located in the kidney with the highest concentration of ETB receptors existing within the medulla(11). Although the role of ETA receptors has been well characterized in the pathophysiology of experimental hypertension, the physiological importance of ETB receptors in modulating sodium excretion and blood pressure regulation in salt-sensitive hypertension is unclear. ETB activation inhibits sodium transport (15, 18-19), and growing evidence suggests a critical role for the renal medullary endothelin system in the integrated response to a high salt intake (6, 20-21). For example, Pollock and others have shown that renal ET-1 production is enhanced in response to one week of a high sodium diet(14), and intramedullary blockade of ETB receptors for 7 days leads to salt-sensitive hypertension. In addition, medullary collecting duct specific knockout of the ETB receptor gene and the ET-1 gene produces salt-sensitive hypertension (1, 6).

Although there is growing evidence from a number of laboratories that the renal endothelin system via ETB receptor activation plays an important role in modulating renal pressure-natriuresis and blood pressure regulation, very little is known about potential abnormalities in renal endothelin system in models of salt-sensitive hypertension. Our laboratory and others have reported that there is a rightward shift in the pressure natriuresis relationship in Dahl salt-sensitive rats (DS) (12). One potential mechanism for the abnormal pressure natriuresis relationship is a defect in the renal medullary endothelin system, resulting from either an abnormality in renal endothelin production or
an altered receptor distribution and signalling. Although several previous studies have reported alterations in renal cortical endothelin production in models of salt-sensitive spontaneous hypertension, the endothelin system was assessed only after long-standing hypertension and renal injury, a condition that is known to stimulate endothelin release (7, 9). Thus, very little is known about potential abnormalities in renal endothelin system in response to increases in sodium intake in the early phases of hypertension in salt-sensitive hypertension models. Therefore, the purpose of this study was to determine if the DS rat has abnormalities in the components of the renal medullary endothelin system when maintained on a high sodium intake for 7 days, prior to the development of hypertension. Another goal of our study was to determine whether chronic intramedullary infusion of ET-1 blunts the hypertensive response to high Na+ intake in DS rats maintained on a high sodium intake for 3 weeks.

**Materials and Methods**

This study was performed using male age matched (8-10 week old), Dahl S and Dahl R rats purchased from Harlan, Inc. (Indianapolis, IN). Animals were housed in a temperature-controlled room (23°C) with a 12:12 hour light/dark cycle. All experimental procedures executed in this study were in accordance with National Institutes of Health guidelines for use and care of animals and approved by the Institutional Animal Care and Use Committee (IACUC) at UMMC.

**Experimental Protocol #1-Effect of 7 days of high salt diet on ET-1 production and receptor expression in Dahl R and Dahl S rats.** Animals were randomly placed into the following groups: Dahl R rats on low Na+ diet (LS, 0.3% NaCl), Dahl R rats on high
Na⁺ diet (HS, 8% NaCl), Dahl S rats on LS, and Dahl S on HS (n=6). The animals were allowed water ad libitum. After six days on respective diets, the animals were placed in metabolic cages for 24 hour urine collection. Urine was collected and on day 7, rats were instrumented with carotid catheters for arterial pressure measurement. On day 8, pressure was measured over a 20 minute period using DataQ systems analysis program and the average over this time period was taken. Immediately following, tissues were collected and snap frozen in liquid nitrogen and stored at -80°C until molecular assays were performed.

**Experimental Protocol # 2: Effect of chronic infusion of ET-1 on blood pressure in Dahl rats:** Male DR and DS (300g) rats were randomly distributed into the following groups: 1) DR vehicle (n=8), 2) DR ET-1(n=8), 3) DS vehicle (n=6), and 4) DS ET-1 (n=5). The rats were maintained on a low Na⁺ diet (0.3% NaCl) prior to experiment. Each rat was uninephrectomized and a chronic indwelling catheter was placed in the medullary interstitium of the other kidney and secured to the renal capsule. Next, the rats were instrumented with a telemetry probe (Data Sciences Int.) for 24 hr blood pressure measurements. The rats were allowed to heal until blood pressure was stabilized. Next, the rats were placed on a high Na⁺ diet, tethered, and infusion of vehicle (saline) or ET-1 (2 ng/kg/min at 0.600 µL/min) was carried out for 15 days.

**Extraction of urinary ET-1.** Equal volume of 20% acetic acid was added to the sample and centrifuged at 3,000 rpm for 10 minutes at 4 °C. A 200mg C₁₈ Sep-Pak was equilibrated with one column reservoir volume (CV) methanol followed by one CV water and one CV 10% methanol. Supernatant was applied to the Sep-Pak column and washed with one CV 10% acetic acid followed by two separate washes with ethyl acetate.
Washes were discarded. Sample was eluted in 3 ml methanol/0.05 M ammonium bicarbonate and collected in polyethylene tubes. Sample was evaporated to dryness using a centrifugal concentrator under a vacuum and reconstituted with assay buffer provided by manufacturer (R&D Systems). Endothelin concentrations were measured using Quantiglo ET-1 ELISA (manufactured by R&D Systems).

**Extraction of Tissue ET-1.** Tissue was snap frozen in liquid nitrogen and stored at -80°C. Frozen samples were pulverized in liquid nitrogen and homogenized for 60 seconds in 10 volumes of 1 M acetic acid containing 10 μg/ml of pepstatin. Samples were heated for 10 minutes at 100°C, placed on ice, and centrifuged at 3500 rpm for 45 min at 4 °C. Supernatant was stored at -80°C and was used for ET-1 quantification. Samples were standardized to total protein using BCA protein kit (Pearce).

**Measurement of urinary and tissue ET-1.** Endothelin concentrations were measured using Quantiglo ET-1 ELISA (R&D Systems) using directions supplied by the company.

**Western Blot Analysis of Endothelin receptors.** Protein was extracted from renal medullary tissue by crunching frozen tissue with a mortar and pestle, then agitation in RIPA buffer containing 10 μL/mL of a protease inhibitor cocktail, PMSF, and sodium orthovanadate (Santa Cruz). Concentration of total protein was acquired using a BCA protein kit (Pearce). Protein lysates (50 μg total protein) were mixed with an equal volume of SDS-Laemmil Sample Buffer (Bio-Rad). Samples were denatured at 95°C for 5 min and chilled on ice for 2 min. They were then loaded into a Criterion gel (BioRad) containing a 4-20% gradient SDS/PAGE gel in running buffer (25mM Tris, 192mM glycine, 0.1% SDS) and ran for 1.5 hours at 120 volts. The gels were then equilibrated in transfer buffer (25mM Tris, 192mM glycine, 20% methanol) for 5 minutes. Next, the
protein was transferred onto a 0.2 µm nitrocellulose membrane in transfer buffer at room
temperature for 2 hours at 40 volts. Membranes were blocked for 1 hour with blocking
buffer (Odyssey). Primary antibody (Rabbit anti ET<sub>B</sub>, Abcam, Cambridge, MA) was
diluted in blocking buffer (1:500) and incubated overnight at 4°C. The membrane was
then washed for 5 min, 3 times. Membranes were then incubated for 45 minutes at room
temperature with gentle agitation in secondary antibody (Donkey anti rabbit conjugated
to IRDye 700DX, 1:5000) diluted in blocking buffer. Blots were then washed 3X in TBS
for 5min per wash. Blots were scanned on an Odyssey infrared imaging system (LiCor)
for detection of the individual fluorophores. The blots were then stripped using NewBlot
western stripping buffer (Licor), washed, and rehybridized using an ET<sub>A</sub> receptor
antibody (Rabbit polyclonal to ET<sub>A</sub> receptor, 1:500, Abcam) using the same secondary as
previously described. After attaining blots for both receptor subtypes, the blots were
rehybridized for β-actin in the same manner as previously described. All ET<sub>B</sub> and ET<sub>A</sub>
bands were normalized to β-actin control bands. Antibodies (Mouse anti β-actin, 1:5000
primary and Donkey anti Mouse conjugated to IRDye 800DX, 1:5000) for β-actin
detection were obtained from Abcam and Rockland, respectively. Quantification of the
western images was carried out with the NIH ImageJ software package.

**Statistical Analysis.** All data are expressed as mean ± SEM. In experiment 1,
comparisons for multigroup analysis were performed by two-way analysis of variance
and by using Tukey’s post-hoc test for comparison between groups. The criterion for
significant differences between groups of study was p<0.05. For experiment 2, a two-
way repeated measures ANOVA was performed, and furthermore, each day was
compared to the baseline measurements at day 4 by Student’s t-test.
Results

To determine if medullary ET-1 plays a role in the development of Dahl S hypertension, we determined urinary ET-1, which is indicative of renal production, in Dahl S and Dahl R rats on low and high Na⁺ diets for one week+, prior to an increase in pressure in the Dahl S. As can be seen in Figure 1 Dahl R rats placed on high Na⁺ diet had a 5-fold increase in ET-1 production (17.8±4 pg/day on NS diet versus 112±44 pg/day on HS, p<.05), however, this increase was significantly blunted in the Dahl S (29.8±5.5 pg/ml HS and 13±2 pg/day NS). Also as indicated in Figure 2, there is a significant reduction in medullary ET-1 in DS rats as compared to DR (70.9±5 DR vs. 30.9±2.8 DS). Finally, our data indicates in figure 3 that DS rats have significantly lower medullary ET₉ receptor expression compared to DR (0.13±0.02 DS on LS and 0.16±.01 DS on HS ET₉/β-actin relative units versus 0.48±0.22 DR on HS and 0.31±0.08 DR on LS ET₉/β-actin relative units, n=6) while also having a significant elevation in ET₈ receptor expression in response to a high salt diet (0.31±0.03 DS on LS versus 0.49±0.10 DS on HS ET₈/β-actin relative units, n=6). The data indicate that a failure of the DS rat to produce ET-1 and a reduction in ET₉ receptor expression in the renal medulla may be important initiating factors in the development of the hypertension observed in this model.

Finally, we wanted to determine if chronic IM infusion of ET-1 would attenuate DS hypertension. As seen in Figure 4, there is a clear trend for a reduction in blood pressure (p=0.11) in response to infusion of ET-1 directly into the renal medulla of DS rats. Furthermore, we found that treatment with ET-1 delayed the onset of hypertension
in response to a high salt intake observed in this model. There was no effect of treatment in DR rats.

**Discussion**

A role for renal medullary derived ET-1 in the control of renal excretory function has accumulated over recent years (1, 8, 21). Multiple studies indicate the importance of this system becomes greater as \( \text{Na}^+ \) intake is increased (21). However, the role of the renal medullary ET-1 system in different forms of genetic and experimental salt sensitive hypertension has yet to be fully elucidated. ET-1 can either elicit a prohypertensive, antinatriuretic effect by activating \( \text{ET}_\text{A} \) receptors in the kidneys (mainly due its cortical actions in the kidney where most of the \( \text{ET}_\text{A} \) receptors are located) or an antihypertensive, natriuretic effect via \( \text{ET}_\text{B} \) receptor activation (mainly in the renal medulla where most of the \( \text{ET}_\text{B} \) receptors are located). Thus, the ability of ET-1 to influence blood pressure regulation and renal function is highly dependent on where ET-1 is produced and which ET receptor type is activated.

The main goal of our study was to determine whether abnormalities in renal medullary ET production (and possibly \( \text{ET}_\text{B} \) receptors levels) play a role in the early phases of hypertension in response to salt in the DS rats. In order to determine if DS rats have altered renal production of ET-1 in the early phases of DS hypertension, we examined the effect of 7 days of high salt diet on urinary ET-1 excretion. It is important to note that only moderate elevations in blood pressure develop after one week on high salt diet in this model (12); therefore, observations in this study were made prior to
elevations in blood pressure. There were two major findings in this study. First, in response to a high salt diet, DR rats had a significant 6-fold increase in urinary ET-1 excretion whereas DS rats only had a 2-fold elevation. Since, urinary ET-1 is indicative of renal production (3), we can conclude that the DS rat has a markedly blunted renal production of ET-1 compared to the DR. Secondly, DS rats had significantly lower tissue levels of ET-1 in the renal medulla than the DR maintained on a high salt intake. This finding is in contrast to a previous study by Barton and colleagues that reported that DS rats have higher levels of ET-1 within the renal medulla in response to a high salt diet (2). There may be multiple reasons for the differences in findings including differences in length of sodium intake (1 week versus 8 weeks), in blood pressure, and methodologies used to measure tissue endothelin levels (2). Collectively, our data indicate that DS rats have a significant reduction in renal medullary ET-1 production in response to a high salt diet in the early phases of DS hypertension.

In addition to a reduction in renal medullary ET-1 production, the DS rat has alterations in renal medullary ET-1 receptor expression during the early phases of DS hypertension. Our data indicates that DS rats have significant reductions in medullary ET₂ receptors as compared to DR rats. Furthermore, in response to a HS diet, medullary ET₁ receptors are upregulated in DS rats. The significance of this depends on which cell type this increase occurs upon. There is evidence that the ET₁ receptor may have natriuretic properties, and can play an adaptive role in the absence of ET₂ receptor function (17). However, if ET₁ receptors were upregulated on vascular cells, this would contribute further to the hypertension in this model, especially with a lack of ET₂ receptors to offset the constrictor properties of ET₁ receptors.
Since we showed that DS rats have significant reductions in renal ET-1 production in response to high salt intake, the next step was to determine the chronic consequences of these effects. Since we proposed that reductions in renal medullary ET-1 contributes to DS hypertension, we hypothesized that chronic infusion of ET-1 directly to the renal medulla would blunt the salt sensitive hypertension in this model by restoring the defect in intramedullary levels of ET-1. Indeed, intramedullary infusion of ET-1 led to a reduction in MAP in DS rats while having no effect in DR rats. While ET-1 did not completely abolish the hypertension long term, intramedullary infusion of ET-1 delayed the onset of hypertension in response to high salt in DS rats because there was no significant change in arterial pressure until day 11, where MAP began to rise equally in both groups. It is hypothesized that this effect is due to the activation of natriuretic ET$_B$ receptors, thus leading to a reduction of blood pressure in the initial phase of Dahl salt-sensitive hypertension. Our data indicates that renal medullary infusion of ET-1 did not completely blunt the increase in pressure in response to a high salt diet in DS rats. There are several potential explanations for this response. For instance, there are many other factors that have been implicated in the pathogenesis of DS hypertension such as decreased synthesis of nitric oxide (10) and 20-Hydroxyeicosatetraenoic acid (23). Thus, intramedullary infusion ET-1 alone would only correct one of the defects responsible for the hypertension and therefore would not be expected to totally prevent the hypertension in DS rats.

While we observed significant increases in urinary ET-1 excretion and renal medullary ET-1 concentration in the DR rats in response to increases in Na$^+$ intake, the
cellular and/or molecular mechanism underlying the defective ET-1 response to Na\(^+\) intake in DS rats remains unknown. ET-1 is thought to be released in response to increased medullary tonicity(25) or tubular flow(16); however, other factors may be involved. Whether medullary tonicity or tubular flow-induced production of ET-1 is altered in DS rats is unclear. Thus, research into the cellular and/or molecular mechanism underlying the defective ET-1 response to Na\(^+\) intake in DS rats warrants further investigation.

While our findings suggest that a decrease in medullary production of ET-1 in the DS rat could play an important role in the early phases in the development of salt-sensitive hypertension observed in the DS rat, several lines of evidence suggest that ET-1 via ET\(_{A}\) receptor activation may contribute to late and established phases of salt-sensitive hypertension. DS rats placed on a high-sodium diet are characterized by attenuated pressure natriuresis, development of hypertension, extensive glomerulosclerosis, renal arteriolar, and tubular injury, as well as progressive renal injury (2, 12). ET-1 levels are increased in the renal cortex of DS rats compared with DR rats and a positive correlation between ET-1 generation in the renal cortex and the extent of glomerulosclerosis has been reported in DS hypertensive rats (2). Also supporting a role of ET-1 in DS hypertension is the finding that acute infusion of a nonselective ET\(_{A}\)-ET\(_{B}\) receptor antagonist directly into the renal interstitium improved renal hemodynamic and excretory function in DS rats but not in DR rats(13). Moreover, chronic blockade of ET\(_{A}\) receptors attenuated the hypertension and proteinuria and ameliorated the glomerular and tubular damage associated with high salt intake in DS rats (2, 12). An important unanswered
question is whether the beneficial effect of the ET blockade in reducing renal injury is mediated through lower blood pressure or through direct renal mechanisms.

In conclusion, we report that the DS rat has several abnormalities in the renal medullary endothelin system when maintained on a high sodium intake during the early phases of DS hypertension. We found that the Dahl salt sensitive rat has an attenuated renal medullary ET synthesis response to a high salt diet during the early phases of DS hypertension. In contrast to the DR rat which had a 6-fold higher urinary endothelin excretion in response to a high Na⁺ diet, urinary endothelin levels increased only 2 fold in DS rats. We also report that medullary endothelin concentration in DS rats on a high Na⁺ diet was also significantly lower than DR rats on a high Na⁺ intake. Finally, DS rats had a significant reduction in medullary ETB receptor expression as compared to DR rats on a high Na⁺ intake. In summary, our data indicate that a decrease in medullary production of ET-1 in the DS rat could play an important role in the early phases of salt sensitive hypertension observed in the DS rat.

**Perspectives and Significance**

While salt sensitive hypertension accounts for a significant portion of human essential hypertension the mechanisms underlying the pathogenesis of this form of hypertension remains to be fully elucidated. The current study suggests that in an experimental model of salt sensitive hypertension, the Dahl salt-sensitive rat, defects in the renal endothelin system may contribute to the pathogenesis of salt-sensitive hypertension. Moreover, our data suggests that the renal medullary ETB receptors may be a potential target for treatment of hypertension. Thus far, clinical studies utilizing ET-1 receptor blockers have shown little effect on blood pressure in hypertensive
patients (22); however, the early ET-1 receptor blockers used were either combined ET\textsubscript{A}/ET\textsubscript{B} blockers or were ET\textsubscript{A} blockers which lacked \textit{in-vivo} specificity (4). Because of the natriuretic and antihypertensive properties of ET\textsubscript{B} receptors, blockade of ET\textsubscript{B} receptors in these studies may have counteracted any beneficial effects of ET\textsubscript{A} receptor blockade. More recently, sitaxsentan, a very specific ET\textsubscript{A} blocker, was shown to be reduce blood pressure and proteinuria in patients with chronic kidney disease (5). Therefore, the importance of ET-1 in human essential hypertension deserves further investigation.

**DISCLOSURE:** The authors declare no conflicts of interest

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Literature Cited


**Figure Legend**

**Figure 1:** Urinary ET-1 excretion is blunted DS rats in response to high Na\(^+\) intake. When DR (n=6) rats are placed on HS diet, urinary ET-1 increases 6-fold, however, the increase in urinary ET-1 excretion was significantly attenuated in Dahl S. * denotes that p<0.05 vs. all other groups.

**Figure 2:** Renal medullary tissue levels of ET-1 were significantly reduced in DS rats, as compared to DR, maintained on either LS (top panel) or HS (bottom panel) diet for 7 days. * denotes that p<0.05 vs. all other groups.

**Figure 3:** A) DS rats have significantly lower ET\(_B\) receptor expression than DR rats. There is a tendency for receptor expression to decrease in DR rats in response to a HS diet; however there is no change in response to changes in dietary salt intake in DS rats. B) In response to a HS diet for one week, there is no significant difference in renal medullary ET\(_A\) receptor expression in DR rats. However, DS rats have a 70 percent increase in ET\(_A\) receptor expression after one week on a HS diet. * denotes p<.05 vs. DS on LS.

**Figure 4:** In response to chronic infusion of ET-1 directly into the renal medulla, the hypertensive response to a high Na\(^+\) diet is delayed in DS rats while there is no effect on Dahl R. * denotes p<0.05 vs. day 4 vehicle infused, # denotes p<0.05 vs. day 4 ET-1 infused.
Figure 1

Urinary Endothelin (pg/day)

- Dahl R
- Dahl S

Legend:
- LS
- HS
Figure 2

Low Salt

High Salt

Medullary ET-1 (pg/mg)

* Dahl R

* Dahl S
Figure 3

A. Medullary ET-B Receptor Expression (ET-B/β-actin relative units)

B. Medullary ET-A Receptor Expression (ET-A/β-actin relative units)