Differential Effect of Renal Cortical and Medullary Interstitial Fluid Calcium on Blood Pressure Regulation in Salt-Sensitive Hypertension

Mildred A. Pointer,1,2 Shaleka Eley,1,2 Lauren Anderson,1,2 Brittany Waters,1,2 Brittany Royall,1,2 Sheena Nichols,1,2 and Candace Wells1,2

BACKGROUND
Hypercalciuria is a frequent characteristic of hypertension. In this report we extend our earlier studies investigating the role of renal interstitial fluid calcium (ISF\textsubscript{Ca}\textsuperscript{2+}) as a link between urinary calcium excretion and blood pressure in the Dahl salt-sensitive (DS) hypertensive model.

METHODS
Dahl salt-sensitive and salt-resistant (DR) rats were placed on control (0.45%) and high (8%) salt diets to determine if changes in renal cortical and medullary ISF\textsubscript{Ca}\textsuperscript{2+} were correlated with changes in urinary calcium excretion and blood pressure.

RESULTS
We observed that renal ISF\textsubscript{Ca}\textsuperscript{2+} was predicted by urinary calcium excretion (P<0.05) in DS rats but not DR rats. Renal cortical ISF\textsubscript{Ca}\textsuperscript{2+} was negatively associated with blood pressure (P<0.03) while renal medullary ISF\textsubscript{Ca}\textsuperscript{2+} was positively associated with blood pressure in DS rats (P<0.04). In contrast, neither urinary calcium excretion nor renal ISF\textsubscript{Ca}\textsuperscript{2+} was associated with blood pressure in the DR rats under the conditions of this study.

CONCLUSION
We interpret these findings to suggest that decreased renal cortical ISF\textsubscript{Ca}\textsuperscript{2+} plays a role in the increase in blood pressure following a high salt diet in salt hypertension perhaps by mediating renal vasoconstriction; the role of medullary calcium remains to be fully understood. Further studies are needed to determine the mechanism of the altered renal ISF\textsubscript{Ca}\textsuperscript{2+} and its role in blood pressure regulation.

Keywords: blood pressure; hypertension; salt-sensitive; urinary calcium.

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Calcium has been implicated in the etiology of hypertension development for many years. Earlier work by investigators nearly 50 years ago showed calcium to be a significant factor in blood pressure regulation.1,2 Subsequent epidemiological studies found that dietary calcium predicts hypertension status3,4 and that dietary calcium supplement can lower blood pressure5-8 especially in salt-sensitive individuals.9,10 The notion that calcium can be both a promoter of hypertension and inhibitor of hypertension has been considered for many years; however, the details of the nature of this dual action is yet to be fully determined.11,12 Our laboratory investigates the underlying mechanism(s) of the anti-hypertensive action of calcium.

Previous studies show that hypercalciuria is a frequent observation in individuals with hypertension13-15; this urinary calcium loss or “leak” appears to account for the elevated parathyroid hormone (PTH) often observed in hypertensives.13,16-19 Similar findings of increased urinary calcium loss following a high salt diet have been observed in animal models of hypertension including the Dahl salt-sensitive (DS) model.20-24 We recently reported that the antihypertensive benefit of hydrochlorothiazide (HCTZ) treatment in the DS hypertensive rat was a result of the calcium sparing effect of HCTZ rather than the sodium loss,24 a finding similar to what has been reported in humans.13,25 Thus, our working hypothesis is that increased urinary calcium loss is a major driver of the rise in blood pressure following a high salt diet in salt-sensitive hypertension.

The mechanism by which urinary calcium loss regulates blood pressure in salt-sensitive hypertension remains to be determined. Renal regulation of calcium excretion is complex. Indeed the kidney has a dual function in regulating extracellular calcium: it acts as a sensor of extracellular calcium and it is a determinant of extracellular calcium levels. Thus, we reason that renal interstitial calcium should reflect both renal calcium sensing and renal calcium reabsorption function in the kidney and, thus, may be important to assess following dietary salt perturbation in an animal model of salt-sensitive hypertension. In this report we extend our earlier studies by investigating whether renal interstitial fluid calcium (ISF\textsubscript{Ca})\textsuperscript{2+}...
is the link between urinary calcium excretion and blood pressure in the DS hypertensive model. Specifically, we wanted to determine whether (i) urinary calcium excretion was associated with renal interstitial calcium; (ii) renal cortical and medullary interstitial calcium was associated with blood pressure; (iii) cortical and medullary interstitial fluid calcium association with blood pressure differed; and (iv) renal interstitial calcium and blood pressure association differed between salt-sensitive and salt-resistant strains. To accomplish these goals we measured renal cortical and medullary ISF$_{\text{Ca}^2+}$ in DS and salt-resistant (DR) rats following 1 week on control (0.45%) or high (8%) salt diet. Although this model of salt-sensitive hypertension may not reflect all of the phenotypic characteristics often observed within humans, it is a model that has been studied extensively in investigating the mechanism of dietary salt-induced hypertension and it is a model used previously that revealed a significant association of urinary calcium and blood pressure.

**MATERIALS AND METHODS**

**Animals and treatments (study protocol)**

Male DS ($n = 33$) and DR ($n = 28$) out bred (John Rapp) rats were purchased (6–8 weeks old) from Harlan Laboratories (Indianapolis, IN) or Charles River. All animals were housed in separate ventilated cages (Animal Care Systems, Littleton, CO) maintained at constant temperature and humidity with automatic 12-hour light cycles. During acclimation animals were maintained on regular chow (Harlan Teklad Laboratories, Madison, WI) and water for a period of 7–10 days. Following acclimation the animals were placed in 1 of 2 treatment groups: regular chow (0.45% NaCl) or high salt (8% NaCl). The Institutional Animal Care and Use Committee of North Carolina Central University approved all protocols used.

**Blood pressure measurement**

Systolic blood pressure was measured by tail-cuff plethysmography (Visitech Systems BP 2000; Apex, NC). Rats were placed in holding units maintained at 37° C. The animals were trained prior to taking an initial blood pressure by placing them into the holding units for approximately 30 minutes each session. The training usually required 2–3 days. A basal blood pressure was obtained over the period of a week; blood pressure measurements were performed again on the last day of the treatment period to determine postintervention blood pressure.

**In situ microdialysis**

Interstitial fluid calcium was determined following the treatment period using the zero net flux method.$^{26}$ Rats were anesthetized using 4% isoflurane (Abbott Laboratories, Chicago, IL) and 70% nitrogen and 30% oxygen gas mix (AirGas, Morrisville, NC) which was delivered at a rate of 0.5 ml/min for the initial induction of anesthesia. Anesthesia was maintained with 2% isoflurane delivered at the same rate as above for the duration of the procedure. Once the animals were completely anesthetized, an incision was made from the lower abdominal region to approximately an inch below the xiphoid process to expose the kidney. A lateral incision was made to expose the left kidney; once the kidney was exposed a 5 mm linear microdialysis probe (Bioanalytical Systems, West Lafayette, IN) was inserted into the cortical region as well as the outer medullary region of the left kidney and set in place using veterinary bonding glue (3M Animal Care Products, St. Paul, MN). Each microdialysis probe was then perfused with 120 mmol/L NaCl in 20 mmol/L HEPES buffer at a rate of 1 µl/min for a 90-minute equilibration period. After the equilibration period, each probe was then perfused at a rate of 1 µl/min with increasing concentrations (0, 0.5, 1.0, 1.5, 2.0, and 3.0 mM) of Ca2+ for 35 minutes followed by a 15-minute collection period. Each sample was collected on ice and stored at 4 °C for later analysis. Probe placement verification was done following sacrifice of the animal. The left kidney was removed and sectioned to visualize the probes. Only those animals with correct probe placement were used for data analysis.

Zero net flux analysis was used to determine renal ISF$_{\text{Ca}^2+}$ concentration. Zero net flux is determined by plotting the difference between the calcium concentration of the dialysate and perfusate (y-axis) vs. the calcium concentration of the perfusate (x-axis). Linear regression analysis was used to determine the best curve or line fit. From this curve the concentration at which the difference between perfusate and dialysate is zero is the concentration of calcium in the interstitial fluid.

**Urine collection**

Overnight urine was collected at baseline and on the evening of the seventh day of the diet. For baseline urines the animals were placed in metabolic cages with free access to water overnight for a period of 10 hours. Animals were then returned to their cages and the collected urine samples were stored at 4 °C for analysis later. On the evening of the seventh day of the treatment, animals were placed in metabolic cages for 10 hours with free access to water. Again, collected urine samples were stored for later analysis.

**Urine calcium measurements**

Urinary calcium concentrations were measured using a colorimetric assay (Calcium Assay kit; (Diagnostic Chemical Limited, Oxford, CT). The calcium assay method was also used to determine the concentration of calcium in the dialysate samples from the in situ microdialysis collections for net zero flux determination.

**Statistical analysis**

Values are expressed as mean ± SEM. Treatment affects (salt effect) were assessed by t-test analysis and linear regression (urinary calcium or ISF$_{\text{Ca}^2+}$ association with blood pressure). The statistical software used was Sigma Stat 3.5 (SPSS, Chicago, IL).
**RESULTS**

**Group characteristics**

There were distinct differences in renal calcium response to dietary sodium between the salt-sensitive and salt resistant rat strains (Table 1). In DS rats, there was urinary calcium loss (Δ $-8.3 \pm 2$) when on 8% salt diet compared to calcium reabsorption (Δ $2.8 \pm 2$ μmoles/10 hours) when on 0.45% salt diet ($P = 0.03, 0.45\%$ vs. 8%; Table 1). There was no difference in urinary calcium response between control (0.45% NaCl) or high salt (8% NaCl) diets in DR rats on. We did observe that DS rats tended to retain calcium when on the control diet while DR rats tended to have a calcium loss ($P < 0.05$).

As expected blood pressure increased in DS rats but not in DR rats following a high salt diet (Δ $29 \pm 3$ on 8% salt vs. Δ $7 \pm 4$ mmHg on 0.45% salt; $P < 0.001$). Finally, renal cortical interstitial fluid calcium (ISFc) was significantly lower in DS rats on a high salt diet compared to DS rats on control salt diet (1.0 ± 0.2 on 0.45% vs. 1.6 ± 0.2 mM on 8% salt diet; $P < 0.001$) but not in DR rats as previously reported by our group. There was no change in renal medullary interstitial fluid calcium (ISFm) in either DS or DR rats following a high salt diet.

**Urinary calcium excretion and blood pressure**

There were also distinct strain differences in urinary calcium excretion association to systolic blood pressure. In Figure 1 (Panel A) the change in urinary calcium excretion from baseline is plotted as the independent variable and post treatment systolic blood pressure as the dependent variable. Negative changes in urinary calcium excretion indicate that urinary calcium increased from baseline measures after the 1-week treatment. Conversely, positive changes in urinary calcium excretion indicated calcium retention from baseline measures. As shown in Figure 1 (Panel A) lower systolic blood pressure is associated with higher urinary calcium retention (positive UcaV values) in DS rats ($r = 0.716; P < 0.01$). In contrast, blood pressure was not significantly associated with urinary calcium in DR rats (Figure 1B; $P = NS$).

**Urinary calcium excretion and renal interstitial calcium**

We next examined whether urinary calcium excretion was associated with renal ISF$_{Ca}^{2+}$ to determine whether renal ISF$_{Ca}^{2+}$ was related to urinary calcium excretion. In Figure 2 we show only the DS rats renal interstitial calcium association to urinary calcium. Panel A shows cortical (ISFc) and panel B show medullary (ISFm) association to change in urinary calcium in DS rats. Both cortical and medullary ISF$_{Ca}^{2+}$ were significantly ($P < 0.05$) associated with urinary calcium excretion DS rats. Higher urinary calcium excretion (negative values) was associated with lower cortical and medullary interstitial calcium. Interestingly, we did not observe this association in DR rats. In fact, we observed no association of change in urinary calcium excretion and interstitial calcium in DR rats (data not shown).

**Renal cortical vs. medullary ISF$_{Ca}^{2+}$ association with blood pressure**

With the finding that urinary calcium excretion was associated with systolic blood pressure and renal interstitial calcium in DS rats, we next wanted to examine whether the ISF$_{Ca}^{2+}$ was associated with blood pressure. We reasoned that such an association would support the idea that renal tubular calcium handling plays a role in systolic blood pressure regulation. As shown in Figure 3 (Panel A) higher cortical interstitial calcium levels were correlated with lower systolic blood pressure in DS rats ($P < 0.03$) while there was no association with systolic blood pressure in DR rats (Panel B). In contrast to cortical ISF$_{Ca}^{2+}$, increased medullary ISF$_{Ca}^{2+}$ was correlated with increased systolic blood pressure in DS rats (Figure 4 Panel A; $P < 0.04$) but no significant association in DR rats (Panel B; NS).

**DISCUSSION**

This study extends our earlier report investigating the relationship between renal ISF$_{Ca}^{2+}$ and blood pressure. Specifically, we show in this report that (i) calcium excretion is associated with lower renal cortical ISF$_{Ca}^{2+}$ and this lower cortical ISF$_{Ca}^{2+}$ is associated with higher blood pressure in DS rats; and (ii) urinary calcium excretion is also associated with lower renal medullary ISF$_{Ca}^{2+}$ but the lower medullary ISF$_{Ca}^{2+}$ is associated with higher systolic blood pressure in DS rats. These results lend further support of our earlier findings that the urinary loss of calcium plays an important part in the rise in blood pressure following a high salt diet. Previous studies by Bukoski and associates have shown that increased extracellular calcium bathing resistance vessels leads to vessel relaxation. Therefore, we interpret our

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**Table 1.** Characteristics of treatment groups

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment</th>
<th>Change in SBP (mmHg)</th>
<th>ISFc [Ca]$^{2+}$ (mM)</th>
<th>ISFm [Ca]$^{2+}$ (mM)</th>
<th>Change in UcaV (μmoles)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dahl SS</td>
<td>0.45% NaCl</td>
<td>3.6 ± 3 (14)</td>
<td>1.6 ± 0.2 (11)</td>
<td>1.1 ± 0.2 (8)</td>
<td>2.8 ± 2 (5)</td>
</tr>
<tr>
<td></td>
<td>8% NaCl</td>
<td>29 ± 3** (19)</td>
<td>1.0 ± 0.2** (19)</td>
<td>1.0 ± 0.2 (7)</td>
<td>−8.3 ± 2* (6)</td>
</tr>
<tr>
<td>Dahl SR</td>
<td>0.45% NaCl</td>
<td>8 ± 3 (16)</td>
<td>1.3 ± 0.1 (17)</td>
<td>1.3 ± 0.1 (7)</td>
<td>−3.8 ± 1*** (5)</td>
</tr>
<tr>
<td></td>
<td>8% NaCl</td>
<td>7 ± 4 (11)</td>
<td>1.1 ± 0.3 (11)</td>
<td>1.1 ± 0.3 (6)</td>
<td>−5.4 ± 2 (6)</td>
</tr>
</tbody>
</table>

Values = mean ± SE; *P < 0.03 compared to 0.45% group; **P < 0.001 compared to 0.45% group; ***P < 0.05 compared to same treatment in Dahl SS. Values in parenthesis represent number of animals in treatment group.

Abbreviations: ISFc, renal cortical interstitial fluid calcium concentration; ISFm, renal medullary interstitial fluid calcium concentration.

*Negative values indicate urinary calcium loss compared to baseline.
results to mean that the rise in blood pressure in DS rats is due in part to impaired compensatory renal vasodilation previously reported to be required for normal maintenance of blood pressure during high salt diet intake\textsuperscript{32–34} by reducing renal cortical ISF \textit{Ca}\textsuperscript{2+} (Figure 5). This is the first report to show that renal interstitial calcium links urinary calcium and blood pressure in salt-sensitive hypertension.

Numerous studies in both humans and animal models of hypertension have demonstrated that urinary calcium excretion is increased following a high salt diet.\textsuperscript{15, 24, 35–38} This association appears to be dependent on a state of salt-sensitivity.\textsuperscript{9, 10, 39} For example, Weinberger \textit{et al.}\textsuperscript{10} observed that calcium supplement was associated with a significant reduction in blood pressure only in African Americans and individuals prone to salt-sensitivity. Our earlier research supports this observation, namely, high salt diet increased urinary calcium excretion only in DS rats not in DR rats.\textsuperscript{24} Additionally, we showed that HCTZ decreased urinary calcium loss in DS rats on a high salt diet and this was associated with the antihypertensive action of HCTZ\textsuperscript{24} but HCTZ had no blood pressure or urinary calcium effect in salt-resistant rats. However, we should note that the association of urinary calcium and blood pressure has been noted in other models of hypertension including genetic (SHR,\textsuperscript{20} stroke prone SHR,\textsuperscript{40} DOCA salt,\textsuperscript{21} and Lyon hypertensive rat)\textsuperscript{40} and volume-dependent\textsuperscript{22} hypertensive models. Thus, it is reasonable to speculate that renal handling of calcium following salt loading is linked to blood pressure regulation in general and not limited to salt-sensitive hypertension.

The findings from this report suggest that the increased urinary calcium excretion is directly associated with decreased renal cortical ISF \textit{Ca}^{2+}, and medullary ISF \textit{Ca}^{2+}; a response not observed in DR rats under similar conditions. Although urinary calcium excretion predicted both cortical and medullary ISF \textit{Ca}^{2+} the association of ISF \textit{Ca}^{2+} to blood pressure differed between the two regions. Cortical ISF \textit{Ca}^{2+} was inversely
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associated with blood pressure while medullary ISF$_{Ca}^{2+}$ was positively associated with blood pressure. This differential renal regional ISF$_{Ca}^{2+}$ association to blood pressure may be a function of regional tubular calcium reabsorption. There is paracellular calcium transport in the proximal tubules and thick ascending limb of Henle but active transcellular calcium transport in the distal tubules. The reabsorption in the proximal thick ascending limb tubular segments is due to an electrochemical gradient driven primarily by sodium reabsorption. The transcellular calcium transport in the distal tubular segment is active, involving calcium transporters such as the transient receptor potential cation channel subtype V member 5, sodium calcium exchanger, and calcium ATPase.

This differential ISF$_{Ca}^{2+}$ association to blood pressure may be a consequence of the anatomical differences between these two regions. The cortical region is highly vascularized, thus, increasing ISF$_{Ca}^{2+}$ in this region could have significant influence on resistance vessel relaxation as observed in mesenteric resistance vessels. Namely, decreasing renal cortical interstitial calcium leads to decreased vessel relaxation and subsequent blood pressure rise following a high salt diet. Previous studies have shown that a lack of compensatory renal vasodilatation contributes to the blood pressure rise following a high salt diet in DS rats. Thus, the rise in blood pressure we observed following a high salt diet may be explained by the decreased extracellular calcium in the highly vascularized cortical region of the kidney.

The outer medullary region (the region we targeted), on the other hand, contains primarily tubular segments such as Figure 3.

Figure 3. Renal cortical interstitial fluid calcium and blood pressure association. Higher renal cortical interstitial fluid calcium (ISFc) is associated with lower blood pressure in in Dahl salt-sensitive rats on control (0.45%) and high salt (8%) diets (top panel). There was no association between ISFc and blood pressure in Dahl salt-resistant rats (bottom panel).

Figure 4. Renal medullary interstitial fluid calcium and blood pressure association. Higher renal medullary interstitial fluid calcium (ISFm) is associated with higher blood pressure in in Dahl salt-sensitive rats on control (0.45%) and high salt (8%) diets (top panel). There was no association between ISFm and blood pressure in Dahl salt-resistant rats (bottom panel).

Figure 5. Scheme of hypercalciuria impact on renal vascular resistance following a high salt diet.
straight distal tubules and thick ascending limbs. Unlike what we found in the cortical region, we observed that higher blood pressure was associated with higher ISF$_{Ca}^{2+}$ levels in the outer medullary region. The mechanism of the positive association of ISF$_{Ca}^{2+}$ and blood pressure is unclear; however, the association may indicate impaired calcium reabsorption due to reduced vasa recta blood flow as reported for salt-sensitive rats. Thus, as the distal tubule, the primary segment for active transcellular calcium reabsorption, reabsorbs calcium the impaired perfusion of this region limits the return of the calcium to the cortical region as blood re-enters the cortical region and, ultimately, limiting the return of calcium to the extracellular compartment.

However, the increased medullary ISF$_{Ca}^{2+}$ due to impaired vasa recta blood flow does not explain the increase urinary calcium excretion seen in DS rats. Alternatively, there may be impaired active distal tubular calcium reabsorption. Proteins involved in active calcium reabsorption in this region of the tubule include transient receptor protein V5, sodium calcium exchanger, and calcium ATPase. Recent reports suggest that African Americans, a group with a propensity for salt-sensitive hypertension, have a greater number of polymorphisms within the transient receptor protein V5 (renal tubular hypertension, have a greater number of polymorphisms calcium reabsorption. Alternatively, there may be impaired active distal tubular calcium reabsorption. Proteins involved in active calcium reabsorption in this region of the tubule include transient receptor protein V5, sodium calcium exchanger, and calcium ATPase. Recent reports suggest that African Americans, a group with a propensity for salt-sensitive hypertension, have a greater number of polymorphisms within the transient receptor protein V5 (renal tubular hypertension, have a greater number of polymorphisms calcium reabsorption. Alternatively, there may be impaired active distal tubular calcium reabsorption. Proteins involved in active calcium reabsorption in this region of the tubule include transient receptor protein V5, sodium calcium exchanger, and calcium ATPase. Recent reports suggest that African Americans, a group with a propensity for salt-sensitive hypertension, have a greater number of polymorphisms within the transient receptor protein V5 (renal tubular hypertension, have a greater number of polymorphisms calcium reabsorption. Alternatively, there may be impaired active distal tubular calcium reabsorption. Proteins involved in active calcium reabsorption in this region of the tubule include transient receptor protein V5, sodium calcium exchanger, and calcium ATPase. Recent reports suggest that African Americans, a group with a propensity for salt-sensitive hypertension, have a greater number of polymorphisms within the transient receptor protein V5 (renal tubular hypertension, have a greater number of polymorphisms calcium reabsorption.

In summary, the results from this study suggest that the rise in blood pressure in salt-sensitive rats following a high salt diet is due, in part, to impaired calcium reabsorption within the kidney. The reduced calcium reabsorption in the cortical region could explain the increased renal vascular resistance observed following a high salt in salt-sensitive hypertension due to impaired renal resistance vessel relaxation. This impaired renal vessel relaxation also contributes to the rise in blood pressure. More studies are required to further characterize the renal calcium reabsorption in the Dahl rat model of salt-sensitive hypertension.

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DISCLOSURE

The authors declared no conflict of interest.

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