Regulation of blood pressure, the epithelial sodium channel (ENaC), and other key renal sodium transporters by chronic insulin infusion in rats

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Song, Jian, Xinqun Hu, Shahla Riazi, Swasti Tiwari, James B. Wade, and Carolyn A. Ecelbarger. Regulation of blood pressure, the epithelial sodium channel (ENaC), and other key renal sodium transporters by chronic insulin infusion in rats. Am J Physiol Renal Physiol 290: F1055–F1064, 2006. First published November 22, 2005; doi:10.1152/ajprenal.00108.2005.—Hyperinsulinemia is associated with hypertension. Dysregulation of renal distal tubule sodium reabsorption may play a role. We evaluated the regulation of the epithelial sodium channel (ENaC) and the thiazide-sensitive NaCl cotransporter (NCC) during chronic hyperinsulinemia in rats and correlated these changes to blood pressure as determined by radiotelemetry. Male Sprague-Dawley rats (~270 g) underwent one of the following three treatments for 4 wk (n = 6/group): 1) control; 2) insulin-infused plus 20% dextrose in drinking water; or 3) glucose water-drinking (20% dextrose in water). Mean arterial pressures were increased by insulin and glucose (mmHg at 3 wk): 98 ± 2 (control), 107 ± 2 (insulin), and 109 ± 3 (glucose), P < 0.01. Insulin (but not glucose) increased natriuretic response to benzamil (ENaC inhibitor) and hydrochlorothiazide (NCC inhibitor) on average by 125 and 60%, respectively, relative to control rats, suggesting increased activity of these reabsorptive pathways. Neither insulin nor glucose affected the renal protein abundances of NCC or the ENaC subunits. However, this is a difficult segment to study and ideal cell lines have yet to be established. Furthermore, insulin may directly increase sodium reabsorption via receptor-mediated activation of specific renal transport proteins.

In situ binding studies using 125I-labeled insulin have revealed that insulin receptors are localized along the entire length of the renal tubule, with the density the greatest in the thick ascending limb and distal convoluted tubule (12, 43). In addition, investigators, utilizing renal micropuncture, have reported that insulin is sodium retentive in the thick ascending limb (30) and/or distal tubule (including thick ascending limb through the collecting duct) (14). Insulin, when provided in the perfusion bath for isolated, perfused tubule studies, has been shown to increase sodium reabsorption in the proximal tubule (3, 48), and the thick ascending limb (20, 24, 33, 48). The ability of insulin to enhance collecting duct sodium reabsorption is less clear. However, A6 cells (toad bladder cells), which are a model for distal tubule cells and express the amiloride-sensitive epithelial sodium channel (ENaC), display increased amiloride-sensitive sodium transport in response to insulin (7). No evidence has been reported for an effect of insulin to stimulate sodium reabsorption in the distal convoluted tubule. However, this is a difficult segment to study and ideal cell lines have yet to be established.

Here, we address the question of whether insulin infusion to induce hyperinsulinemia affects protein abundance, cellular distribution, or activity of specific renal sodium transport proteins and channels of the distal convoluted tubule through the collecting duct. We also determine whether chronic infusion of insulin will result in elevated blood pressure in these same animals. We utilize radiotelemetry to continuously monitor blood pressure over a 4-wk period in response to insulin infusion. We use a novel approach to assess specific sodium channel and transporter subunit activities in vivo by measuring the rats’ natriuretic responses to selective sodium transporter and channel blockers. Finally, we utilize immunoblotting and immunoperoxidase labeling to examine the renal abundance of target proteins.

INRINSIN IS ANTINATRIURETIC. Early studies by Atchley et al. (2) demonstrated that discontinuation of an insulin infusion to diabetic patients resulted in a brisk natriuresis. Similarly, Miller and Bogdonoff (36) showed that infusion of insulin into normal subjects resulted in a reduction in urinary sodium excretion. More detailed studies by DeFronzo et al. (13, 14) demonstrated that, in both dogs (14) and humans (13), insulin increased sodium reabsorption by the kidney as well as reduced sodium excretion independently of blood glucose levels, filtered load of glucose, glomerular filtration rate, renal blood flow, and plasma aldosterone levels.

Hyperinsulinemia has also been linked to hypertension in humans and animals. Semichronic infusion of insulin (anywhere from 5 to 14 days) has been demonstrated to increase blood pressure modestly in male rats (8, 10, 22, 25, 26, 28). Urepression of the sympathetic nervous system (22), the renin-angiotensin system (8), thromboxane synthesis (28), and endothelin-1 (26) has all been implicated as playing a role. Some of the above factors influence vascular tone, which most likely affects blood pressure. However, in addition, many of these factors may influence sodium reabsorption by the kidney. Furthermore, insulin may directly increase sodium reabsorption via receptor-mediated activation of specific renal transport proteins.

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and cellular distribution of the following proteins: the thiazide-sensitive Na–Cl cotransporter (NCC or TSC) and the α-, β-, and γ-subunits of ENaC. We also examine protein abundance of two selected kinases that play important roles in distal tubular sodium reabsorption, i.e., serum- and glucocorticoid-regulated kinase-1 (SGK-1), a regulator of ENaC activity (27), and WNK4 (“with no lysine”) kinase, a regulator of NCC activity (53).

METHODS

Animals, study design, and diets. Eighteen male Sprague-Dawley rats (250–280 g) were obtained from Taconic Farms (Gaithersburg, MD). After a brief, 3- to 4-day equilibration period, all rats were implanted with radiotelemetric blood pressure transmitters (Data Sciences International, St. Paul, MN). Under isoflurane anesthesia, the pressure-sensitive tip of the fluid-filled catheter of the radio transmitter was advanced into the aorta via an incision in the femoral artery. The body of the radio transmitter was secured into a pouch under the skin near the left hindlimb. Blood pressure was measured for 10 s at 10-min intervals for the entire study. After a 5- to 7-day recovery/baseline period, rats were randomly assigned to one of the following three treatment groups (n = 6/group): 1) control, 2) insulin-infused, or 3) glucose water-drinking. A second surgery was done at this time to subcutaneously implant osmotic minipumps (Alzet model 2002, Alza, Palo Alto, CA) into insulin-infused rats. Other rats received sham surgery. Human insulin (Humulin-R, Eli Lilly, Indianapolis, IN) diluted with water was infused at a rate of 20 U/kg body wt -1·day -1. The insulin pump was replaced at 2 wk with a new pump in case of insulin delivery failure, affinity purification, and characterization of the polyclonal antibodies against NCC, α-, β-, and γ-ENaC have been previously described (45, 46). WNK4 was a rabbit anti-human polyclonal antibody obtained from Alpha Diagnostic (San Antonio, TX), and SGK-1 was a sheep polyclonal obtained from Upstate Biotechnology (Lake Placid, NY).

Immunohistochemistry. Separate animals were set up for immunohistochemical examination of the ENaC subunits and NCC. The left kidney was processed in paraffin, and 5-μm sections were cut. Heat-induced target retrieval was performed using pH 6 citrate buffer (Zymed Laboratories) to unmask antigenic sites. Endogenous peroxidase activity was stopped by incubation with 2% H2O2 for 20 min. Tissues were incubated with the primary antibody (1:1,000), i.e., α-, β-, or γ-ENaC or NCC, overnight at 4°C. An Envision+ System (DakoCytomation, Carpinteria, CA) goat anti-rabbit antibody was used to conduct peroxidase labeling. Then, 3,3′-diaminobenzidine tetrachloride dihydrate was applied for 10 min and the tissue was counterstained with Mayer’s hematoxylin to allow anatomic definition. A positive reaction was identified as a brown stain in the cytoplasm or a dark brown/black nuclear stain as a result of superimposition of the 3,3′-diaminobenzidine tetrachloride dihydrate reaction and the blue counterstain. Pictures were taken with a Photometrics Cool Snap camera (Scananalytics, Fairfax, VA) mounted to a Nikon Eclipse E600 microscope with a ×100 oil-immersion lens for a total magnification of ×1,000.

Statistics. Data were evaluated by SigmaStat (Chicago, IL). One-way ANOVA followed by Tukey’s multiple comparisons test was used to determine significant differences between means.

RESULTS

Physiological effects of insulin infusion. There were no significant differences in measured sodium intake and total measured calories consumed per body weight or in final body weight due to treatment, although there was a trend for insulin-infused rats to be heavier (Table 1). In fact, there was a
significant increase in weight gain in the insulin-infused rats. Interestingly, kidney weight normalized to body weight was significantly lower in the insulin-infused and glucose-drinking rats. Finally, blood glucose levels measured at the end of the study were not different between the treatments.

**Plasma components.** Sodium and potassium concentrations in the plasma were not significantly different due to treatment (Table 2). As expected, plasma insulin was increased by insulin infusion. Aldosterone and renin activity was not affected by treatment. There were no significant differences in triglyceride concentrations between groups.

**Blood pressure changes.** In a preliminary study in which rats were fed equivalent diets and sodium intake was reduced by ~50% in insulin-infused and glucose-drinking rats (see METHODS), we observed no differences between treatments for mean arterial blood pressure (MAP). However, in this study MAP was significantly elevated in the both the glucose water-drinking group and the insulin-infused group, relative to control rats, after 16 or 20 days, respectively (Fig. 1). The increase was between 5 and 10 mmHg. There were no differences between the glucose-drinking and the insulin-infused groups, however, at any time point.

**Response to selective natriuretic agents.** Sodium excretion in response to a single administration of selective natriuretic agents was used as a novel test of specific transporter or channel activities. Responses to benzamil and HCTZ are shown in Fig. 2, A and B, respectively. We plotted data as absolute sodium excretion because baseline urinary excretion for sodium was not different. The natriuretic response to both agents was observed in the first 3-h urine collection. The insulin-infused rats excreted 125 and 60% more sodium in response to benzamil and HCTZ, respectively (a significant increase), relative to control rats, possibly indicating greater in vivo activity of ENaC and NCC. These tests were repeated in a separate set of animals for the insulin-infused and control

### Table 1. Physiological effects of insulin infusion

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Insulin</th>
<th>Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium intake, mmol/kg</td>
<td>2.97±0.18</td>
<td>3.15±0.38</td>
<td>2.94±0.18</td>
</tr>
<tr>
<td>Water/fluid intake, ml/kg</td>
<td>94±13</td>
<td>140±20</td>
<td>157±20</td>
</tr>
<tr>
<td>Dry food intake, g/kg</td>
<td>55.0±4.0</td>
<td>35.5±4.2*</td>
<td>27.5±2.2*</td>
</tr>
<tr>
<td>Kidney weight, g/kg</td>
<td>270±16</td>
<td>339±22</td>
<td>330±13</td>
</tr>
<tr>
<td>Blood glucose, mmol/l</td>
<td>391±11</td>
<td>432±5*</td>
<td>399±16</td>
</tr>
<tr>
<td>Triglycerides, mmol/l</td>
<td>126±11</td>
<td>170±5*</td>
<td>137±11</td>
</tr>
<tr>
<td>Aldosterone, pmol/l</td>
<td>3.32±0.05</td>
<td>2.96±0.10*</td>
<td>2.98±0.11*</td>
</tr>
<tr>
<td>Renin activity, ng/ml</td>
<td>6.77±0.33</td>
<td>6.66±0.61</td>
<td>6.77±0.16</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6/group for all comparisons. Sodium, water/fluid, dry food, and kcal intakes are averages of measurements made at 6, 13, and 20 days. BW, body wt. *Significantly (P < 0.05) different from the control mean, as determined by 1-way ANOVA followed by Tukey’s multiple comparisons test.

### Table 2. Final plasma determinations

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Insulin</th>
<th>Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium, mmol/l</td>
<td>142±1</td>
<td>143±2</td>
<td>144±1</td>
</tr>
<tr>
<td>Potassium, mmol/l</td>
<td>5.5±0.1</td>
<td>5.3±0.1</td>
<td>5.4±0.1</td>
</tr>
<tr>
<td>Insulin, pmol/l</td>
<td>390±60</td>
<td>705±105*</td>
<td>375±45</td>
</tr>
<tr>
<td>Aldosterone, pmol/l</td>
<td>275±72</td>
<td>355±178</td>
<td>230±100</td>
</tr>
<tr>
<td>Renin activity, ng/ml</td>
<td>59±16</td>
<td>74±15</td>
<td>59±10</td>
</tr>
<tr>
<td>Triglycerides, mmol/l</td>
<td>1.18±0.19</td>
<td>1.13±0.20</td>
<td>1.42±0.13</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6/group for all comparisons. Renin activity is expressed as generated angiotensin I. *Significantly (P < 0.05) different from the control mean and †from the glucose-drinking mean, as determined by 1-way ANOVA followed by Tukey’s multiple comparisons test.
treatments (n = 7/group). In this case, we confirmed an increase in sodium excretory response in the insulin-infused rats relative to the control rats in response to both benzamil and HCTZ. Insulin-infused rats excreted 145 and 76% more sodium in response to HCTZ and benzamil, respectively, than did control rats (P < 0.012).

**ENaC subunits.** In Fig. 3, we show representative immunoblots from the whole kidney, cortex, outer, and inner medullary homogenates probed with anti-α-, β-, or γ-ENaC antibodies. A summary of densitometric statistics are found in Tables 3 (α- and β-ENaC) and 4 (γ-ENaC). An equal amount of total protein (20–30 μg) was loaded in each lane of each blot. In the cortex and whole kidney homogenates, there were no significant differences between any of the groups for the densities of any of the ENaC channel subunit bands. However, in the inner medulla, β-ENaC was modestly but significantly decreased in the insulin-infused rats relative to the glucose water-drinking rats; and in the outer and inner medulla, the lower (70-kDa band) associated with γ-ENaC was increased in the glucose water-drinking rats relative to control.

**ENaC immunohistochemistry.** Because we found an increase in natriuretic activity in response to benzamil, yet very little change in ENaC subunit protein abundances, we elected to examine subcellular localization of the subunits, because trafficking to the apical membrane, most likely to increase activity, has been demonstrated (32, 34). Representative immunoperoxidase labeling of α-ENaC in the cortex (top), outer medulla (middle), and inner medulla (bottom) is shown in Fig. 4. Labeling for α-ENaC was fairly intense in the collecting duct principal cells of the cortex and outer medulla in all treatments. However, lesser labeling was observed in the inner medullary collecting ducts. Staining for α-ENaC was more apical in the rats treated with insulin, especially in the cortex (top, middle column), whereas the staining appeared more diffuse in the control rats (top, left column).

In Fig. 5, we show immunoperoxidase labeling for β-ENaC. Like α-ENaC, β-ENaC labeling appeared most intense near the apical membrane of the principal cells in the cortex in insulin-infused rats. Lesser labeling was observed in the outer medullary collecting duct for all treatments.

Representative immunoperoxidase-labeling of γ-ENaC is shown in Fig. 6. γ-ENaC labeling was found in all regions, and the stain was most intense in the apical membrane of insulin-infused and glucose-drinking rats. Again, the labeling pattern was more diffuse in control rats.

**NCC.** In Fig. 7, representative immunoblots probed with our NCC antibody loaded with whole kidney or cortex homogenates (A and C) and bar graph summaries (B and D) are shown. NCC abundance was not significantly affected by treatment.

Table 3. **Summary of immunoblot densitometry for α- and β-ENaC**

<table>
<thead>
<tr>
<th>Protein/Region</th>
<th>Control ± SE</th>
<th>Insulin ± SE</th>
<th>Glucose ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-ENaC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole kidney</td>
<td>100 ± 7</td>
<td>100 ± 10</td>
<td>116 ± 6</td>
</tr>
<tr>
<td>Cortex</td>
<td>100 ± 9</td>
<td>103 ± 10</td>
<td>93 ± 6</td>
</tr>
<tr>
<td>Outer medulla</td>
<td>100 ± 5</td>
<td>115 ± 11</td>
<td>122 ± 3</td>
</tr>
<tr>
<td>Inner medulla</td>
<td>100 ± 5</td>
<td>97 ± 3</td>
<td>108 ± 5</td>
</tr>
<tr>
<td>β-ENaC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole kidney</td>
<td>100 ± 2</td>
<td>103 ± 2</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>Cortex</td>
<td>100 ± 7</td>
<td>103 ± 12</td>
<td>109 ± 8</td>
</tr>
<tr>
<td>Outer medulla</td>
<td>100 ± 6</td>
<td>96 ± 11</td>
<td>110 ± 13</td>
</tr>
<tr>
<td>Inner medulla</td>
<td>100 ± 3</td>
<td>88 ± 7*</td>
<td>108 ± 3</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6/group for all comparisons. ENaC, epithelial sodium channel. *Significantly (P < 0.05) different from the glucose-drinking mean, for that protein, as determined by 1-way ANOVA followed by Tukey’s multiple comparisons test.

Table 4. **Summary of immunoblot densitometry for γ-ENaC (both bands)**

<table>
<thead>
<tr>
<th>Protein (Band)/Region</th>
<th>Control ± SE</th>
<th>Insulin ± SE</th>
<th>Glucose ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ-ENaC (85 kDa)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole kidney</td>
<td>100 ± 4.2</td>
<td>100 ± 4</td>
<td>100 ± 6</td>
</tr>
<tr>
<td>Cortex</td>
<td>100 ± 2.6</td>
<td>97 ± 3</td>
<td>92 ± 3</td>
</tr>
<tr>
<td>Outer medulla</td>
<td>100 ± 4.9</td>
<td>111 ± 9</td>
<td>109 ± 11</td>
</tr>
<tr>
<td>Inner medulla</td>
<td>100 ± 11</td>
<td>119 ± 7</td>
<td>132 ± 14</td>
</tr>
<tr>
<td>γ-ENaC (70 kDa)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole kidney</td>
<td>100 ± 4</td>
<td>96 ± 7</td>
<td>116 ± 7</td>
</tr>
<tr>
<td>Cortex</td>
<td>100 ± 5</td>
<td>108 ± 5</td>
<td>106 ± 8</td>
</tr>
<tr>
<td>Outer medulla</td>
<td>100 ± 16</td>
<td>132 ± 12</td>
<td>207 ± 36*</td>
</tr>
<tr>
<td>Inner medulla</td>
<td>100 ± 14</td>
<td>112 ± 14</td>
<td>205 ± 42*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6/group for all comparisons. *Significantly (P < 0.05) different from the control mean, as determined by 1-way ANOVA followed by Tukey’s multiple comparisons test.
Figure 8 shows representative immunoperoxidase labeling in a control rat, an insulin-infused rat, and a glucose water-drinking rat. We saw exclusive labeling of the apical portion of the distal convoluted tubule cells in all treatments. No discernible differences in expression level or cellular distribution were found.

WNK4 and SGK-1. Finally, to more comprehensively address the mechanism underlying the observed greater response to the ENaC- and NCC-selective natriuretic agents in the insulin-infused rats, we elected to look at the protein abundances of two regulatory proteins, i.e., SGK-1, shown to regulate ENaC trafficking (27), and WNK4 kinase, shown to reduce NCC activity (53). In Fig. 9A, we show sample immunoblots for SGK-1. Clearly, no significant differences were found for this protein. WNK4 is shown in Fig. 9B. The specific band for this protein runs at ~155 kDa (51). We found a modest but significant decrease in this band in cortex homogenates in the insulin-infused rats relative to controls ($P < 0.05$). The same trend was seen in whole kidney homogenates; however, the difference was not significant ($P = 0.076$).

**DISCUSSION**

In the present study, we have demonstrated that long-term infusion of insulin causes a modest but significant increase in MAP in male Sprague-Dawley rats. In general, this study is in agreement with several others (10, 16, 25, 35, 49) showing an increase in MAP in rats infused with insulin. However, unlike others, we used continuous radiotelemetry to record blood pressure, whereas most others used tail-cuff recording or direct intra-arterial readings by an externalized in-dwelling catheter. Additional stimulation of the sympathetic nervous system due to stress surrounding tail-cuff recording or tethering using the externalized catheter method might have affected the outcome in some of the other studies (likely to exacerbate responses), especially during hyperinsulinemia, a model that has been demonstrated to have elevated sympathetic activity associated with it (1, 22, 37, 42). Therefore, our animal model is unique in that we were able to probe the role of sodium transporters in vivo in a chronically instrumented rat in which MAP has been measured continuously over the length of the study.

Also, in agreement with others (23, 40) we showed that the consumption by rats of a diet high in a simple sugar, i.e.,
glucose in the drinking water, also raises blood pressure, in our case, to a similar degree as the insulin infusion. This is likely due to the development of insulin resistance in these rats (4, 18, 23). We did not plan to address the degree of insulin resistance in this study. Nevertheless, it is important to consider the possibility that the hypertension caused by the insulin infusion may have also been due to the development of insulin resistance rather than to a direct response to the insulin. Insulin has been demonstrated to increase Ca\(^{2+}\)/H\(_{1001}\)-ATPase activity (54) and endothelial nitric oxide release (47) from endothelial tissue, thus resulting in vasodilation in sensitive tissues, phenomena expected to lower, not raise, blood pressure. In line with this, Hall and associates (11, 21) have consistently demonstrated that dogs, which do not as readily develop insulin resistance when infused with physiological levels of insulin, fail to show an increase in blood pressure, even in the presence of reduced kidney mass and high sodium intake, which should render the animal highly susceptible to hypertensive stimuli.

These data in combination with our preliminary work also suggest that the increase in blood pressure observed with insulin infusion was “salt sensitive,” because no differences in blood pressure were observed between groups when dietary sodium intake of the insulin-infused and glucose-drinking rats was only half that of the control group (see RESULTS, Blood pressure changes). This was contrary to Brands et al. (9), who showed no differences in the rise in MAP in rats given anywhere from 0.6 to 11.4 meq Na\(^+\)/day while being infused intravenously for 7 days with ~2.5 U insulin·kg body wt\(^{-1}\)·day\(^{-1}\). However, it is in agreement with Tomiyami et al. (49), who showed increased MAP with insulin infusion in salt-sensitive, but not salt-resistant, Dahl rats, and only when they were fed a high-salt diet. Differences in study length, achieved plasma insulin levels, blood glucose levels, and other unknown variables may have resulted in these different findings.

Despite similarly increased MAP between insulin-infused and glucose-drinking rats, only rats receiving the insulin infusion had an increased natriuretic response to benzamil (a selective ENaC inhibitor) and HCTZ (a selective Na-Cl co-transporter inhibitor). This may imply that the mechanisms underlying the increase in blood pressure between these two treatments are different. Perhaps insulin infusion increases distal tubule sodium reabsorption associated with it and glucose drinking does not. A variation of this approach, termed “pharmacological blockade or knockout” of sodium channel or transporter activity, was employed by Frindt et al. (19) using

![Immunoperoxidase labeling of β-ENaC](https://via.placeholder.com/150)

**Fig. 5.** Immunoperoxidase labeling of β-ENaC. β-ENaC was localized on tissue with our own specific antibody against the β-ENaC subunit (45, 46). Shown are examples of staining in the (top to bottom) cortical, outer medullary, and inner medullary collecting ducts (×1,000 magnification) from a control (left), insulin-infused (middle), and glucose-drinking rat (right). Labeling was most intense in the apical cell membranes in the cortex of insulin-infused rats.
amiloride (rather than benzamil) to block ENaC and polythiazide to block NCC. In those studies, the drugs were given to assess the contribution of ENaC vs. NCC reabsorption of sodium in rats that were on a sodium-deprived diet. However, the use of such agents to gauge the activity of these specific transport routes is quite novel and thus may be subject to skepticism. The fact that the natriuretic response to each of these agents was positive (relative to baseline), different (between the treatment groups), appropriate to expectations (with regard to degree of response), and reproducible among different groups of rats indicates that pharmacological blockade may be a valuable means of identifying sites of altered transport in

Fig. 6. Immunoperoxidase labeling of γ-ENaC. Shown are examples of staining in the (top to bottom) cortical, outer medullary, and inner medullary collecting ducts (×1,000 magnification) from a control (left), insulin-infused (middle), and glucose-drinking rat (right). γ-ENaC labeling was found in all regions, with increased apical residency in the cortex of insulin-infused and glucose-drinking rats.

Fig. 7. Thiazide-sensitive Na-Cl cotransporter (NCC) immunoblotting and bar graph summary. Representative blots and bar graph of whole kidney (A and B) and cortex homogenates (C and D) are shown. Each lane was loaded with an equal amount of total protein from a different rat’s sample (n = 6/treatment). Blots were probed with our own anti-NCC antibody (45, 46). No significant differences were observed between treatments for band density.
the kidney. Nevertheless, it is important to also consider the possibility that differences in volume status, rate of drug absorption from the intraperitoneal cavity, and/or distribution throughout the body might exist between treatments and thus might also affect the response.

Insulin has been shown to increase sodium reabsorption through ENaC in A6 cells and cortical collecting duct cell lines via activation, likely phosphorylation, of SGK-1 (7, 39, 41, 50). However, whether insulin may also change the protein abundances of individual subunits of ENaC, as do aldosterone (34) and vasopressin (15), is not known. In this study, when sodium levels were matched, we found that a chronic yet modest elevation in circulating levels of insulin did not dramatically affect the protein abundances of any of the subunits, nor did it affect the ratio of the 70- to 85-kDa band of γ-ENaC as does aldosterone (34). Furthermore, NCC abundance, also shown to be increased by aldosterone (29), was not significantly increased by insulin infusion or glucose drinking in this study.

On the other hand, we did see increased apical residency of α-, β-, and γ-ENaC in the cortical collecting ducts from the insulin-infused rats relative to control. This occurred despite no measurable change in the final circulating level of plasma aldosterone in these rats (Table 2). Aldosterone has also been shown to increase apical residency of ENaC (32, 34). Therefore, the increase in ENaC on the apical membrane may have been the cause of the increased benzamil responsivity in these rats. In addition, it might have played a role in the elevated blood pressure; however, an absolute causal relationship between these variables could not be determined from this study.

However, inappropriately high ENaC or NCC activity has been associated with hypertension in humans with Liddle’s or Gordon’s syndrome, respectively (31, 51).

In addition, WNK4 kinase has been shown to inhibit NCC by phosphorylating the transporter (52). It may also suppress its delivery to the plasma membrane (53). We found a significant decrease in the density in WNK4 on the immunoblot for the insulin-infused rats relative to the glucose water-drinking rats. This may explain the differential response of these two groups to HCTZ. However, we did not find clear discernible differences in cellular distribution of NCC with immunoperoxidase-based labeling.

Furthermore, it is possible that if we had achieved a relatively higher level of circulating insulin via the infusion, we might have observed a significant effect on ENaC and NCC abundances. However, this study would necessitate colinfusion of glucose to control hypoglycemia. Based on our preliminary work, this is difficult to titrate, stressful to the animals in a chronic scenario, and results in weight loss in the animals. Unlike aldosterone or vasopressin, which we can manipulate over fairly high ranges in the circulation to study their physiological roles in renal transport, high levels of insulin, in an insulin-sensitive rat can rapidly lead to hypoglycemia and death.

On the other hand, insulin-resistant rat models such as the obese Zucker rat have 2- to 10-fold higher circulating levels of insulin (5, 6). In these animals, we found an increase in the abundance of the β-subunit of ENaC as well as NCC. However, additional studies will need to be done to determine

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Fig. 8. Immunoperoxidase labeling of NCC. NCC was localized on tissue with our own specific antibody against NCC (45, 46). Shown are examples of staining in the distal convoluted tubule (×1,000 magnification) from a control (left), insulin-infused (middle), and glucose-drinking rat (right). Labeling was very strong and apically localized to the distal convoluted tubules in all three treatments. No clear differences were discernible.

Fig. 9. Serum- and glucocorticoid-regulated kinase-1 (SGK-1) and “with no lysine” kinase (WNK4) immunoblots. Shown are representative blots for SGK-1 (A) and WNK4 (B) of whole kidney (top) and cortex (bottom) homogenates, respectively. Each lane was loaded with an equal amount of total protein from a different rat’s sample (n = 6/treatment). Blots were probed with either a sheep polyclonal antibody against SGK-1 (Upstate Biotechnology; A) or a rabbit polyclonal antibody against WNK4 (Novus Biologicals; B). *Mean band density is significantly different (P < 0.05) from that of the control group by 1-way ANOVA followed by Tukey’s multiple comparisons test.
whether the insulin receptors in the kidney become “resistant” as do peripheral insulin receptors in these rats.

Overall, chronic insulin infusion as well as drinking of a glucose solution were shown to increase blood pressure in rats when dietary sodium intakes were carefully matched. This increase corresponded to, and thus may have been the result of, increased activity of distal renal tubular sodium transport pathways including NCC and ENaC, possibly via trafficking into the apical membrane.

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