Upregulation of Na\(^+\) transporter abundances in response to chronic thiazide or loop diuretic treatment in rats

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Furosemide and hydrochlorothiazide (HCTZ) are diuretics commonly used in clinical practice. Furosemide exerts its diuretic action by binding to the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter in the thick ascending limb and blocking ion transport (34). HCTZ exerts its diuretic action by binding to the Na\(^+\)-Cl\(^-\) cotransporter (NCC) in the distal convoluted tubule (4). Increased Na\(^+\) delivery to the distal nephron may result in enhanced Na\(^+\) absorption downstream from the distal site of diuretic action. Recent research in the pathophysiology of other disorders of water and electrolyte balance suggests that long-term adaptive mechanisms may be important in these disorders and may involve altered expression of transporter proteins, such as aquaporin water channels and Na\(^+\) transporters (13, 19, 21, 22, 30). In this context, it appears possible that diuretic resistance produced by long-term diuretic administration is in part due to adaptive increases in the abundances of Na\(^+\) transporters in the distal nephron and collecting ducts.

Therefore, this study was undertaken to elucidate the molecular basis of the adaptive mechanisms in long-term use of diuretics. We hypothesized that a secondary increase in distal delivery of Na\(^+\) may induce compensatory changes in the abundance of Na\(^+\) transporters downstream from the primary site of the diuretic action. To test this hypothesis, we administered either furosemide or HCTZ for 7 days to rats and investigated the effects on the expression of Na\(^+\) transporter proteins by using semiquantitative immunoblotting and immunohistochemistry on tissue from rat kidneys.

DIURETICS ARE FREQUENTLY PRESCRIBED for treatment of hypertension and edematous disorders. Although they are clinically useful to induce negative Na\(^+\) balance, diuretic resistance is often encountered to limit their further use. Diuretic resistance may be explained in part by increased Na\(^+\) absorption in downstream renal tubule segments related to increased Na\(^+\) delivery (10).

Furosemide and hydrochlorothiazide (HCTZ) are diuretics commonly used in clinical practice. Furosemide exerts its diuretic action by binding to the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter (NKCC2) in the thick ascending limb and blocking ion transport (34). HCTZ exerts its diuretic action by binding to the Na\(^+\)-Cl\(^-\) cotransporter (NCC) in the distal convoluted tubule (4). Increased Na\(^+\) delivery to the distal nephron may result in enhanced Na\(^+\) absorption downstream from the distal site of diuretic action. Recent research in the pathophysiology of other disorders of water and electrolyte balance suggests that long-term adaptive mechanisms may be important in these disorders and may involve altered expression of transporter proteins, such as aquaporin water channels and Na\(^+\) transporters (13, 19, 21, 22, 30). In this context, it appears possible that diuretic resistance produced by long-term diuretic administration is in part due to adaptive increases in the abundances of Na\(^+\) transporters in the distal nephron and collecting ducts.

Therefore, this study was undertaken to elucidate the molecular basis of the adaptive mechanisms in long-term use of diuretics. We hypothesized that a secondary increase in distal delivery of Na\(^+\) may induce compensatory changes in the abundance of Na\(^+\) transporters downstream from the primary site of the diuretic action. To test this hypothesis, we administered either furosemide or HCTZ for 7 days to rats and investigated the effects on the expression of Na\(^+\) transporter proteins by using semiquantitative immunoblotting and immunohistochemistry on tissue from rat kidneys.

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METHODS

Animals and experimental protocols. Specific pathogen-free male Sprague-Dawley rats (170–230 g; SLC, Shizuoka, Japan) were placed in metabolism cages 3 days before the beginning of the study. Control and treated rats were designated randomly, and all were provided with a daily, fixed amount of finely ground regular rat chow (18 g·200 g body wt$^{-1}·$day$^{-1}$) and two separate bottles of drinking water, one containing 0.8% NaCl and 0.1% KCl and the other containing tap water. All the rats ate all of the offered rat chow and showed a steady increase in body weight throughout the study period. The chronic studies were carried out as follows.

Chronic furosemide infusion. Rats were anesthetized with enflurane (Choongwae Pharma, Seoul, Korea), and osmotic minipumps (model 2ML1, Alzet, Palo Alto, CA) were subcutaneously implanted to deliver 12 mg/day of furosemide (Handok, Seoul, Korea). Furosemide was dissolved in a 1.7% ethanolamine solution. Control rats were implanted with the minipumps containing vehicle (ethanolamine) alone.

Chronic HCTZ infusion. Rats were infused with either 3.75 mg/day of HCTZ (YuHan, Seoul, Korea) or vehicle for 7 days. These infusions were achieved by using the same vehicle solution (1.7% ethanolamine) and osmotic minipumps as described above.

Physiological measurements. During the course of the studies, daily urine was collected to evaluate responses to the diuretics. Urine osmolality was measured with a cryoscopic osmometer (Osmomat 030-D-M, Gonotec, Berlin, Germany), and urine electrolytes and creatinine were measured with an ion-selective method (System E4A, Beckman Coulter, Fullerton, CA). Serum samples were collected at the time each rat was killed for determination of the serum aldosterone concentration by radioimmunoassay (SPAC-S aldosterone kit, Daiichi Pharmaceutical, Tokyo, Japan).

Semiquantitative immunoblotting. After 7 days of infusion, the rats were killed by decapitation, and kidneys were rapidly removed and placed in chilled isolation solution containing 250 mM sucrose, 10 mM triethanolamine (Sigma, St. Louis, MO), 1 μg/ml leupeptin (Sigma), and 0.1 mg/ml phenylmethylsulfonyl fluoride (Sigma) titrated to pH 7.6. Next, the kidneys were dissected to obtain cortex and inner stripe of the outer medulla. Each region was separately homogenized in 10 (cortex) or 1 ml (outer medulla) of ice-cold isolation solution at 15,000 rpm with three strokes for 15 s with a tissue homogenizer (PowerGun 125, Fisher Scientific, Pittsburgh, PA). After homogenization, total protein concentration was measured by using the bicinchoninic acid protein assay reagent kit (Sigma) and adjusted to 2 μg/ml with isolation solution. The samples were then stabilized by the addition of 1 vol 5× Laemmli sample buffer/4 vol sample, heated to 60°C for 15 min.

Initially, “loading gels” were done on each sample set to allow fine adjustment of loading amount to guarantee equal loading on subsequent immunoblots. Five micrograms of protein from each sample were loaded into each individual lane and electrophoresed on 12% polyacrylamide-SDS mini-gels by using Mini-PROTEAN III electrophoresis apparatus (Bio-Rad, Hercules, CA) and then stained with Coomassie blue dye (0.025% solution made in 4.5% methanol and 1% acetic acid; G-250, Bio-Rad). Selected bands from these gels were scanned (GS-700 Imaging Densitometry, Bio-Rad) to determine density (Molecular Analyst version 1.5, Bio-Rad) and relative amounts of protein loaded in each lane. Finally, protein concentrations were “corrected” to reflect these measurements. For immunoblotting, the proteins were transferred electrophoretically from unstained gels to nitrocellulose membrane (Bio-Rad). After being blocked with 5% skim milk in PBS-T (80 mM Na$_2$PO$_4$, 20 mM NaH$_2$PO$_4$, 100 mM NaCl, and 0.1% Tween-20, pH 7.5) for 30 min, membranes were probed overnight at 4°C with the respective primary antibodies. For probing blots, all antibodies were diluted into a solution containing 150 mM NaCl, 50 mM sodium phosphate, 10 mg/dl sodium azide, 50 mg/dl Tween 20, and 0.1 g/dl bovine serum albumin (pH 7.5). The secondary antibody was goat anti-rabbit IgG conjugated to horseradish peroxidase (31458, Pierce, Rockford, IL) diluted to 1:3,000. Sites of antibody-antigen reaction were viewed by using enhanced chemiluminescence substrate (ECL RPN 2106, Amersham Pharmacia Biotech, Buckinghamshire, UK) before exposure to X-ray film (Hyperfilm, Amersham Pharmacia Biotech).

Immunohistochemistry. The kidneys were perfused by retrograde perfusion via the abdominal aorta with 1% PBS to remove blood and then with periodate-lysine-paraformaldehyde (0.01 M Na$_2$O$_4$, 0.075 M lysine, and 2% paraformaldehyde in 0.375 M Na$_2$HPO$_4$ buffer, pH 6.2) for kidney fixation for 3 min. After completion of perfusion, each kidney was sliced into 5-mm-thick pieces and immersed in 2% periodate-lysine-paraformaldehyde solution overnight at 4°C. Each kidney was dehydrated with a graded series of ethanol and embedded in polyester wax. The embedded pieces of kidney were sectioned at 5-μm thickness on a microtome (RM 2145, Leica Instruments, Nussloch, Germany).

The sections were dewaxed with a graded series of ethanol and treated with 3% H$_2$O$_2$ for 30 min to eliminate endogenous peroxidase activity. They were blocked with 6% normal goat serum (S-1000, Vector Laboratory, Burlingon, CA) for 15 min. They were incubated overnight at 4°C with the respective primary antibodies diluted in PBS. After incubation, they were washed with PBS and incubated for 30 min in biotinylated goat anti-rabbit IgG (BA-1000, Vector Laboratory) at room temperature. Next, peroxidase standard vector ABC kit (PK-4000, Vector Laboratory) was added for 30–60 min at room temperature. The sections were washed with PBS and incubated in 3,3′-diaminobenzidine substrate kit (SK-4100, Vector Laboratory). Hematoxylin staining was used as a counterstain. The slides were mounted with Canvaid balsam.

Primary antibodies. For semiquantitative immunoblotting and immunohistochemistry, we used previously characterized polyclonal antibodies. Affinity-purified polyclonal antibodies against the thick ascending limb NKCC2 (9), thiazide-sensitive NCC (20), and α-, β-, and γ-subunits of the epithelial Na⁺ channel (ENaC) (26) were used. In addition, the present study utilized polyclonal antibodies against aquaporin-1 (AQP1) (40), AQP2 (32), and AQP3 (8).

Statistics. Values were presented as means ± SE. Quantitative comparisons between the groups were made by Mann-Whitney U-test (Statview software, Abacus Concepts, Berkeley, CA). To facilitate comparisons in the semiquantitative immunoblotting, we normalized the band density values by dividing by the mean value for the control group. Thus the mean for the control group is defined as 100%. $P < 0.05$ was considered as indicative of statistical significance.

RESULTS

Chronic furosemide infusion. Physiological data from urine collections confirmed the effects of furosemide. Table 1 shows urinary data on the final day of chronic furosemide infusion. Urine output was markedly increased and urine osmolality was decreased by furosemide infusion compared with vehicle-infused controls. In furosemide-infused rats, urinary Na⁺ and Cl⁻ excretion was remarkably increased, but there was no signifi-
significant change in urinary K⁺ excretion. Creatinine clearance was also not significantly affected by furosemide infusion.

Despite persistent osmotic diuresis for 7 days, body weight measured before euthanasia was not different between the two groups (furosemide-infused rats, 198 ± 4 g, vs. vehicle-infused rats, 204 ± 4 g). Furthermore, serum aldosterone level was not altered by furosemide infusion (furosemide-infused rats, 0.40 ± 0.15 nM, vs. vehicle-infused rats, 0.38 ± 0.14 nM).

Analysis of the renal cortical homogenates prepared for immunoblotting revealed a significant increase in total amount of protein in furosemide-infused rat cortices compared with vehicle-infused controls (80.8 ± 3.7 vs. 66.0 ± 3.5 mg/100 g body wt, P < 0.05). This finding is consistent with the previously demonstrated furosemide-induced cortical hypertrophy (17, 18). On the other hand, there was no difference in total amount of protein in the outer medullary homogenates between the two groups.

Figure 1A shows an immunoblot of the thick ascending limb NKCC2 from cortical homogenates. Furosemide infusion for 7 days resulted in a significant increase in NKCC2 abundance in the cortex. Normalized band densities for furosemide-infused and vehicle-infused rats were 151 ± 10 and 100 ± 10, respectively (means ± SE, P < 0.05). In Fig. 1C, the abundance of NKCC2 in the outer medulla was also increased by furosemide infusion (122 ± 5 for furosemide-infused rats vs. 100 ± 3 for vehicle-infused rats, P < 0.01). Parallel Coomassie blue-stained SDS-polyacrylamide gels demonstrated uniform loading among all samples (Fig. 1, B and D), ruling out the possibility that the increase in band density in cortex or outer medulla could be due to differences in loading.

The abundance of thiazide-sensitive NCC in the cortex was not altered by furosemide infusion (Fig. 2). Normalized band densities for furosemide-infused rats vs. vehicle-infused rats were 101 ± 11 vs. 100 ± 19, respectively. Figure 3 shows NCC immunohistochemistry from the cortex of furosemide-infused and vehicle-infused rats. Consistent with previous studies (12, 17), distal tubular hypertrophy was noted in furosemide-infused rats. However, no difference in NCC immunoreactivity was found between furosemide-infused and vehicle-infused rats.

Figure 4 shows the immunoblots carried out for the three ENaC subunits in cortical homogenates. Significant increases in all three subunits of ENaC protein abundance were found in response to chronic furosemide infusion. Normalized band densities of each subunit protein were as follows: α-subunit (Fig. 4A), 187 ± 25 for furosemide vs. 100 ± 22 for vehicle, P < 0.05; β-subunit (Fig. 4B), 155 ± 8 for furosemide vs. 100 ± 15 for vehicle, P < 0.05; and γ-subunit (Fig. 4C) 168 ± 16 for furosemide vs. 100 ± 9 for vehicle, P < 0.05.)

![Fig. 1. Effects of furosemide infusion on abundance of thick ascending limb Na⁺-K⁺-2Cl⁻ cotransporter (NKCC2). Immunoblots of cortical homogenates (A) and outer medullary homogenates (C) from Sprague-Dawley rats receiving a subcutaneous infusion of furosemide (12 mg/day) or vehicle via osmotic minipump are shown.](image1)

![Fig. 2. Effect of furosemide infusion on abundance of thiazide-sensitive Na⁺-Cl⁻ cotransporter (NCC) in cortex. Immunoblot of the same cortical homogenates from Sprague-Dawley rats as used for Fig. 1 is shown. Each lane was loaded with a protein sample from a different rat. The blot was probed with rabbit polyclonal anti-NKCC2 antibody L224. A: blot of cortex was loaded with 10 μg total protein/lane. Band density for NKCC2 protein was significantly increased by furosemide infusion. C: blot of outer medulla was loaded with 5 μg total protein/lane. Band density for NCC protein was significantly increased by furosemide infusion. Twelve percent Coomassie blue-stained SDS-polyacrylamide gels from cortical homogenates (B) and outer medullary homogenates (D) confirm equal loading among lanes.](image2)
0.05. Responses of the ENaC subunits in the outer medulla of the same rats were qualitatively the same as those seen in the cortex (Fig. 5). Band density of each subunit protein was as follows: α-subunit (Fig. 5A), 171 ± 27 for furosemide vs. 100 ± 17 for vehicle, P < 0.05; β-subunit (Fig. 5B), 986 ± 91 for furosemide vs. 100 ± 33% for vehicle, P < 0.01; and γ-subunit (Fig. 5C), 242 ± 24 for furosemide vs. 100 ± 22 for vehicle, P < 0.01.

Figure 6 demonstrates ENaC immunohistochemistry from the renal cortex of furosemide-infused and vehicle-infused rats by using the β-ENaC antibody. Figure 6, top, shows low-power images demonstrating a marked increase in immunostaining in furosemide-infused vs. vehicle-infused rat. In higher-power images (Fig. 6, bottom), the size of the tubular epithelial cells appeared larger in a furosemide-infused rat compared with a vehicle-infused rat. Staining conditions and exposure settings on the microscope were identical for the two images. Similar observations were made in three pairs of rats.

We also carried out immunoblotting for AQP1–3 in the cortex and outer medulla (not shown). In the cortex, there were no significant changes in the band densities of AQP1 (105 ± 7 for furosemide vs. 100 ± 7 for vehicle), AQP2 (149 ± 18 for furosemide vs. 100 ±
and AQP3 (126 ± 17 for furosemide vs. 100 ± 12 for vehicle). As in the cortex, band density for each aquaporin protein in the outer medulla was not significantly altered by furosemide infusion (AQP1, 80 ± 9 vs. 100 ± 7; AQP2, 94 ± 11 vs. 100 ± 7; and AQP3, 72 ± 12 vs. 100 ± 22).

**Chronic HCTZ infusion.** Chronic infusion of HCTZ revealed similar changes in urinary indices to furosemide infusion. Table 2 shows urinary data on the final day of HCTZ infusion. HCTZ produced a significant increase in urine output and a decrease in urine osmolality and sodium excretion.

### Table 2. Urinary data from chronic hydrochlorothiazide infusion

<table>
<thead>
<tr>
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<th>Vehicle</th>
<th>HCTZ</th>
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<tbody>
<tr>
<td>Volume, ml/day</td>
<td>13 ± 1</td>
<td>34 ± 4*</td>
</tr>
<tr>
<td>Osmolality, mosmol/kg/H2O</td>
<td>1,738 ± 32</td>
<td>952 ± 36*</td>
</tr>
<tr>
<td>Na+, meq/day</td>
<td>3.3 ± 0.6</td>
<td>7.5 ± 0.9†</td>
</tr>
<tr>
<td>K+, meq/day</td>
<td>3.8 ± 0.3</td>
<td>4.2 ± 0.1</td>
</tr>
<tr>
<td>Cl−, meq/day</td>
<td>4.3 ± 0.9</td>
<td>8.9 ± 1.1†</td>
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<tr>
<td>Creatinine clearance, ml/min</td>
<td>1.36 ± 0.11</td>
<td>1.53 ± 0.09</td>
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</table>

Values are means ± SE; n = 6 rats/group. HCTZ, hydrochlorothiazide. *P < 0.01, †P < 0.05.

Fig. 5. Effects of furosemide infusion on abundance of ENaC in outer medulla. Each lane was loaded with outer medullary homogenate from a different rat. A: blot was loaded with 30 μg total protein/lane and was probed with rabbit polyclonal anti-α-ENaC antibody L766. B: blot was loaded with 30 μg total protein/lane and was probed with rabbit polyclonal anti-β-ENaC antibody L558. C: blot was loaded with 30 μg total protein/lane and was probed with rabbit polyclonal anti-γ-ENaC antibody L550. Band density for each subunit protein was significantly increased by furosemide infusion.

Fig. 6. Immunohistochemistry of β-ENaC in cortex from Sprague-Dawley rats infused with vehicle or furosemide. Each panel shows a marked increase in immunostaining in furosemide-infused vs. vehicle-infused rat. Top, ×100; bottom, ×400.
osmolality. As in the furosemide infusion study, urinary Na\(^+\) and Cl\(^-\) excretion were elevated but K\(^+\) excretion was not significantly increased in HCTZ-infused rats. Creatinine clearance was not affected.

In the HCTZ infusion study, the total amount of cortical protein measured in the homogenates of cortex dissected from the kidneys of HCTZ-infused rats was not different from the corresponding value of vehicle-infused rats (68.6 ± 2.4 vs. 65.2 ± 1.5 mg/100 g body wt). The two groups had no difference in total amount of protein in the outer medullary homogenates.

Figure 7A shows an NCC immunoblot in cortical homogenates from vehicle-infused and HCTZ-infused rats. The abundance of NCC protein in HCTZ-infused rats was significantly increased compared with vehicle-infused rats (normalized band densities: 166 ± 14 for HCTZ vs. 100 ± 13 for vehicle, \(P < 0.05\)). Figure 7B shows a loading control for the immunoblot, achieved by running an identically loaded polyacrylamide gel and staining it with Coomassie blue. There was little or no variation in the densities of the major bands stained with Coomassie blue, demonstrating that the increase in NCC band density seen in Fig. 7A was not due to unequal loading.

Figure 8 shows NCC immunohistochemistry from the cortex of HCTZ-infused and vehicle-infused rats.

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**Fig. 7.** Effect of hydrochlorothiazide (HCTZ) infusion on abundance of thiazide-sensitive NCC in cortex. Immunoblot of cortical homogenates from Sprague-Dawley rats receiving a subcutaneous infusion of HCTZ (3.75 mg/day) or vehicle via osmotic minipump is shown. Each lane was loaded with protein sample from a different rat. A: blot was loaded with 10 \(\mu\)g total protein/ lane and was probed with rabbit polyclonal anti-NCC antibody L573. Band density for NCC protein was significantly increased by HCTZ infusion. B: 12% Coomassie blue-stained SDS-polyacrylamide gel from cortical homogenates, confirming equal loading among lanes.

**Fig. 8.** Immunohistochemistry of thiazide-sensitive NCC in cortex from Sprague-Dawley rats infused with vehicle or HCTZ. An increase in immunostaining is noted in HCTZ-infused vs. vehicle-infused rat. Top, \(\times 100\); bottom, \(\times 400\).
Compatible with the immunoblot result, it reveals an increase in immunostaining in an HCTZ-infused vs. a vehicle-infused rat. Similar observations were made in three pairs of rats.

Figure 9 shows the immunoblots from the same cortical homogenates for the three ENaC subunits. In response to HCTZ infusion, an increase in β-ENaC protein abundance was noted (Fig. 9B). Normalized band density increased to 139 ± 7 of vehicle-infused controls (100 ± 4, P < 0.05). However, no significant increase was seen in the abundance of the α-subunit (Fig. 9A; 111 ± 13 for HCTZ vs. 100 ± 11 for vehicle) or γ-subunit (Fig. 9C; 122 ± 10 for HCTZ vs. 100 ± 12 for vehicle). Changes in the abundance of ENaC in the outer medulla are depicted in Fig. 10. Band density of α-ENaC was not significantly increased by HCTZ infusion (Fig. 10A; 122 ± 19 for HCTZ vs. 100 ± 9 for vehicle). In contrast, both β-ENaC (Fig. 10B; 201 ± 28 for HCTZ vs. 100 ± 16 for vehicle, P < 0.05) and γ-ENaC (Fig. 10C; 212 ± 17 for HCTZ vs. 100 ± 8 for vehicle, P < 0.01) protein abundance were increased in the outer medullary homogenates. A parallel Coomassie blue-stained SDS-polyacrylamide gel demonstrated uniform loading among all samples (Fig. 10D), ruling out the possibility that the increase in band density in the outer medulla could be due to differences in loading.

Figure 11 demonstrates ENaC immunohistochemistry from the cortex of HCTZ-infused and vehicle-infused rats by using the β-ENaC antibody. Compatible with the immunoblot result, it shows an increase in immunostaining in an HCTZ-infused vs. a vehicle-infused rat. Similar observations were made in three pairs of rats.

**DISCUSSION**

In this study, we analyzed changes in the abundance of the major apical Na⁺ transporters in the distal sites of the renal tubule in response to chronic infusion of a loop diuretic (furosemide) or a thiazide diuretic (HCTZ) in rats. Table 3 summarizes the percent changes in NKCC2, NCC, and three subunits of ENaC protein abundances with either diuretic treatment. The data provide important new information relevant to clinical use of these diuretics and address an important physiological issue: What is the effect of increased distal fluid delivery on transporter abundances? The main results showed that the Na⁺ channel expressed in connecting tubule and collecting duct, ENaC, was increased in its abundance in response to chronic infusion of either diuretic, consistent with previous reports showing increased Na⁺ absorption in these segments (14, 38). In addition, furosemide induced an increased
abundance of NKCC2, whereas HCTZ induced an increased abundance of NCC. The important clinical implication from our study is that the diuretic tolerance associated with long-term diuretic use may be caused by increases in the abundance of distal Na\(^+\)/H\(^+\) transporters.

**Chronic diuretic infusion induces compensatory increase in ENaC abundance.** Both furosemide and HCTZ caused a substantial diuresis and natriuresis, although the response was greater with furosemide than with HCTZ. With concomitant salt and water substitution, the animals gained body weight steadily without apparent volume depletion during the experiment. On the basis of the known sites of action of the diuretics, it is likely that Na\(^+\)/H\(^+\) delivery to collecting duct was increased by both diuretics.

ENaC constitutes the rate-limiting step for Na\(^+\)/H\(^+\) reabsorption in the connecting tubule and collecting duct in the rat kidney (6, 16, 36). Because ENaC is a heteromultimeric protein formed by the association of three homologous subunits, α, β, and γ (2), we tested its expression by using the specific polyclonal antibodies to each subunit (26). In our study, chronic furosemide abundance of NKCC2, whereas HCTZ induced an increased abundance of NCC. The important clinical implication from our study is that the diuretic tolerance associated with long-term diuretic use may be caused by increases in the abundance of distal Na\(^+\) transporters.

**Table 3. Percent changes in Na\(^+\) transporter abundances in response to chronic furosemide or hydrochlorothiazide infusion**

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<tr>
<th></th>
<th>Furosemide</th>
<th>HCTZ</th>
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<tr>
<td><strong>Cortex</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NKCC2</td>
<td>151 ± 10†</td>
<td>ND</td>
</tr>
<tr>
<td>NCC</td>
<td>101 ± 11</td>
<td>166 ± 14†</td>
</tr>
<tr>
<td>α-ENaC</td>
<td>187 ± 25†</td>
<td>111 ± 12</td>
</tr>
<tr>
<td>β-ENaC</td>
<td>155 ± 8†</td>
<td>139 ± 7§</td>
</tr>
<tr>
<td>γ-ENaC</td>
<td>168 ± 16†</td>
<td>122 ± 10</td>
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<tr>
<td><strong>Outer medulla</strong></td>
<td></td>
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</tr>
<tr>
<td>NKCC2</td>
<td>122 ± 5§</td>
<td>ND</td>
</tr>
<tr>
<td>α-ENaC</td>
<td>171 ± 27†</td>
<td>122 ± 19</td>
</tr>
<tr>
<td>β-ENaC</td>
<td>986 ± 91*</td>
<td>201 ± 28†</td>
</tr>
<tr>
<td>γ-ENaC</td>
<td>242 ± 24*</td>
<td>212 ± 17*</td>
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Values are means ± SE. NKCC2, Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter; NCC, Na\(^+\)-Cl\(^-\) cotransporter; ENaC, epithelial Na\(^+\) channel; ND, not done. The band density values were normalized by dividing by the mean value for the vehicle-infused control group. *P < 0.01 vs. vehicle-infused controls. †P < 0.05 vs. vehicle-infused controls.
infusion increased the protein abundance of all three subunits in the cortex and more prominently in the outer medulla. Chronic HCTZ infusion also increased the abundance of β-subunit in the cortex and β- and γ-subunits in the outer medulla. Although regulation of ENaC activity in the collecting duct and connecting tubule involves several control processes, such as regulated trafficking (16, 25), and posttranslational modifications, such as methylation (34), it is likely that an important element of ENaC regulation is achieved through control of subunit abundance as seen in response to aldosterone or vasopressin (7, 26). We did not investigate the mechanism of altered ENaC subunit protein abundance in the present study, although regulation of mRNA levels (31, 39) and regulation of ENaC protein half-life (5, 27, 41) have been described in previous studies.

Our animal protocol of salt and water substitution was apparently effective in preventing extracellular fluid volume depletion, as evidenced by the absence of an increase in serum aldosterone level. Furthermore, NCC abundance, which is regulated by aldosterone (20), was not altered by chronic furosemide administration. Thus it appears unlikely that the changes in ENaC subunit abundance are mediated by either aldosterone or vasopressin. Another factor previously shown to alter ENaC subunit abundance is acid-base state. Alkali loading was demonstrated to increase both β- and γ-ENaC abundance in rat kidney (19), raising the possibility that the increases in the abundance of these two subunits could be related to the metabolic alkalosis seen with chronic diuretic administration.

On the basis of prior literature, it appears quite plausible that the increase in ENaC subunit expression seen with chronic diuretic administration is related to the effects on the delivery of NaCl and water to the collecting duct. Specifically, in furosemide-infused, volume-replaced rats, Stanton and Kaissling (18, 38) demonstrated an increase in distal Na⁺ absorption accompanied by an increase in epithelial cell volume of the distal convoluted tubule, connecting tubule, and collecting duct. Consistent with a general hypertropic effect of chronic furosemide infusion, we found that the total amount of protein in cortical homogenates was larger in furosemide-infused rats compared with vehicle-infused controls. These findings seem to be compatible with a previous study (23) showing that furosemide infusion with high-salt intake caused an increase in DNA synthesis in distal segments downstream of the thick ascending limb. The mechanism by which the renal tubule cells sense an increase in flow rate is unknown but may involve the central cilium present in renal tubule epithelial cells (33).

An increase in Na⁺-K⁺-ATPase activity in the cortical collecting duct was shown after either furosemide (37) or HCTZ (14) administration. Although the mechanism is unknown, the increase in basolateral Na⁺-K⁺-ATPase activity would reduce intracellular Na⁺, promoting Na⁺ influx via apical ENaC in the collecting duct. A regulatory role of altered intracellular and extracellular ion concentrations on ENaC function has been reviewed (15), and it appears likely that intracellular Na⁺ activity has effects on ENaC that are independent of effects on the electrochemical driving forces for Na⁺ ion movement through the channel. These effects could possibly include altered abundance of ENaC subunit proteins. Although changes in transporter abundance are by no means the only way in which transport capacity of renal tubule cells is regulated, the increase in ENaC abundance that we have observed is likely to represent adaptive responses to allow for the conservation of Na⁺ in response to diuretic administration.

Abundance of NKCC2 and NCC is increased by furosemide and HCTZ infusion, respectively. Chronic furosemide infusion significantly increased the abundance of NKCC2 in cortex and outer medulla, and chronic HCTZ infusion markedly increased the abundance of NCC in cortex. This may represent a compensatory response and may contribute to diuretic resistance. Consistent with the effect of HCTZ administration on NCC abundance, a previous study demonstrated that the binding density of [³H]metolazone, an indirect measure of NCC abundance, was increased by chronic HCTZ infusion (3, 29) despite a decrease in NaCl transport capacity of distal convoluted tubule (11). The distal convoluted tubules from HCTZ-infused rats preserved epithelial structure and unipolar apical staining when they were examined by the NCC immunohistochemistry (Fig. 8). These findings are in contrast to the report of Loffing et al. (24) that thiazide (especially metolazone) treatment in Wistar rats was associated with injury of distal convoluted tubule cells, a basal shift of NCC localization, and a significant decrease in mRNA transcripts of NCC (24).

We do not know whether the discrepancies result from differences in animals (Sprague-Dawley vs. Wistar rats) and doses (3.75 mg·rat⁻¹·day⁻¹ in the present study vs. 40 mg·kg body wt⁻¹·day⁻¹) of HCTZ.

Another interesting finding is that NCC abundance was not increased by furosemide infusion. Previous micropuncture experiments in rat kidneys have demonstrated that chronic furosemide infusion increases Na⁺ reabsorption in the distal tubule (12, 38), an effect that is perhaps related to increased transport via ENaC. However, in rats, chronic furosemide infusion increased binding of [³H]metolazone to renal cortical membranes, suggesting an increase in NCC abundance (3). Recently, Abdallah et al. (1) reported that furosemide infusion increased NCC protein abundance, but this effect was blocked by spironolactone, suggesting that the response was dependent on a rise in plasma aldosterone concentration. Compared with our study, they used furosemide in much larger doses (125 mg·kg⁻¹·day⁻¹), leading to a 10-fold increase in daily urine volume. Our finding that NCC abundance was not increased by furosemide infusion may be related to the lack of an increase in serum aldosterone in our study. In addition, other neurohormonal factors can be considered as contributing to the changes in NCC protein abundance during furosemide infusion. Prostaglandin É₂ production can be stimulated by furosemide
administration (28), and nitric oxide generation can be increased by high-salt intake (42). The possibility that these factors might act to affect NCC protein abundance needs to be investigated in future studies.

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