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Loop Diuretic Infusion Increases Thiazide-Sensitive Na^{+}/Cl^{-}-Cotransporter Abundance: Role of Aldosterone

JOSEPH G. ABDALLAH,* ROBERT W. SCHRIER,* CHARLES EDELSTEIN,* SUSAN D. JENNINGS,* BRUCE WYSE,*† and DAVID H. ELLISON*†

*Division of Nephrology and Hypertension, University of Colorado School of Medicine and Veterans Administration Medical Center, Denver, Colorado; and †Oregon Health Sciences University, Portland, Oregon.

Abstract. Chronic infusion of loop diuretics into animals induces structural and functional changes in the distal nephron. These changes include increases in the activity of the thiazide-sensitive Na^{+}/Cl^{-}-cotransporter (NCC). The NCC was recently demonstrated to be an aldosterone-induced protein. These experiments were designed to test the hypotheses that chronic loop diuretic infusion, with replacement of NaCl losses, increases NCC protein abundance and that this effect results, in part, from stimulation by aldosterone. Sprague-Dawley rats received vehicle (group 1), furosemide (22 mg/100 g body wt per d) (group 2), or furosemide plus spironolactone (22 and 20 mg/100 g body wt per d, respectively) (group 3). Urine output was higher for groups 2 and 3 than for group 1 (151 ± 32, 149 ± 24, and 12 ± 4 ml, respectively; $P < 0.0001$). Immunoblot analysis of NCC protein demonstrated that loop diuretics increased NCC protein abundance by nearly 100% (from 2562 ± 30 to 5248 ± 151 arbitrary units, $P < 0.01$). Spironolactone decreased NCC protein abundance by 66% (to 3532 ± 113 units), compared with the furosemide-treated group ($P < 0.005$). Northern blot analysis of NCC mRNA demonstrated no significant effect of furosemide (NCC/glyceraldehyde-3-phosphate dehydrogenase ratios: group 1, 0.6 ± 0.12; group 2, 0.5 ± 0.05; $P > 0.05$, NS) These results indicate that increased NCC activity during chronic loop diuretic infusion is associated with increases in NCC protein abundance. A portion of the furosemide effect can be prevented by blockade of mineralocorticoid receptors.

The Na^{+}/Cl^{-}-cotransporter (NCC) (SLC12A3) of the distal convoluted tubule (DCT) reabsorbs 5 to 10% of filtered sodium chloride. Disruption of NCC function by gene mutation leads to Gitelman's syndrome, an autosomal recessive disorder characterized by salt wasting, hypokalemic alkalosis, hypomagnesemia, low calcium excretion, and low BP (1). Conversely, NCC activity can be stimulated by adrenal steroid hormones and by chronically administered loop diuretics (2), thus contributing to diuretic resistance.

Diuretics are commonly used to treat patients with kidney disease, hypertension, and edema. Resistance to loop diuretics occurs frequently, and strategies to prevent or reverse resistance continue to be sought. Chronic loop diuretic infusion leads to hypertrophy and hyperplasia of DCT cells (3), together with an increase in NCC activity (4,5), an increase in the number of binding sites for the thiazide-like drug metolazone (6,7), and an increase in Na^{+}/K^{+}-ATPase activity (8). There is evidence that several factors acting together may contribute to the structural and functional changes that occur during chronic loop diuretic administration. First, chronic diuretic infusion increases distal NaCl delivery (4). Second, chronic diuretic infusion stimulates several neurohormonal systems. Although Stanton and Kaissling (5,9) demonstrated that structural and functional changes in the DCT can occur during loop diuretic infusion even if circulating levels of glucocorticoids, mineralocorticoids, and vasopressin are maintained constant, an important role of hormonal stimulation remains possible. We previously demonstrated that dietary NaCl restriction, which is a potent stimulus of aldosterone secretion, increased the thiazide-sensitive component of Na^{+} transport by the DCT (4). Subsequent experiments demonstrated that chronic aldosterone administration to adrenalectomized rats increased the number of binding sites for metolazone (6,10) and increased the transport capacity of the DCT (10). We also demonstrated that DCT cells express both mineralocorticoid receptors and 11β-hydroxysteroid dehydrogenase, indicating that these cells are targets of aldosterone action (11). Kim *et al.* (12) recently demonstrated that dietary salt restriction or exogenous mineralocorticoid administration increases the abundance of NCC protein in the kidney cortex, which indicates that the NCC is an aldosterone-induced protein. Beck *et al.* (13) demonstrated that the morphologic response to chronic administration of loop diuretics could be altered by angiotensin-converting enzyme inhibition. The experiments presented here tested the hypotheses that (*1*) chronic loop diuretic administration increases NCC activity at least in part by increasing the abundance of the transport

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Correspondence to Dr. David H. Ellison, Suite 262, Oregon Health Sciences University, 3314 SW US Veterans Hospital Road, Portland, OR 97201. Phone: 503-494-8490; Fax: 503-494-5330; E-mail: ellisond@ohsu.edu

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protein and (*2*) circulating aldosterone contributes importantly to this effect.

Materials and Methods

Animals

Male Sprague-Dawley rats (200 to 250 g; Harlan Sprague-Dawley, Indianapolis, IN) were divided into three groups. Group 1 animals (control) received a constant infusion of vehicle (50% DMSO) via an osmotic minipump (Alzet model 2ML1; Alza Corp., Palo Alto, CA) implanted subcutaneously under methoxyflurane anesthesia. The animals were allowed free access to standard rat chow (Ralston Purina Co., St. Louis, MO) and tap water. Group 2 animals (furosemide infusion) received a constant subcutaneous infusion of furosemide (22 mg/100 g body wt per d; furosemide was dissolved in 50% DMSO, with NaOH to maintain the pH between 7 and 8) via an osmotic minipump and were allowed free access to standard chow and a saline drinking solution (0.9% NaCl, 0.1% KCl), to prevent severe volume and potassium depletion, as described previously (9). Group 3 animals (furosemide and spironolactone infusion) received a constant infusion of furosemide at the same dose as for group 2. In addition, spironolactone pellets (Innovative Research of America, Sarasota, FL) were implanted subcutaneously 1 d before osmotic pump placement. The pellets delivered 20 mg of spironolactone/100 g body wt per d. Group 3 animals were also allowed free access to standard chow and the saline drinking solution described above. Body weight was measured before pellet and pump placement and at the end of the experiment, 8 d later. An additional group of furosemide-treated animals were euthanized after 3 d, and their kidneys were prepared for Northern analysis (as described below).

All study protocols were approved by the Animal Subjects Committee at the Veterans Administration Medical Center of Denver. Animals were housed in the animal care facility.

Urine Volume and Composition

Urine was collected daily in metabolic cages. Sodium and potassium levels were measured by flame photometry (Instrumentation Laboratory, Lexington, MA). Osmolality was measured by vapor pressure osmometry (5500; Wescor, Logan, UT). Rates of urinary sodium and potassium excretion were calculated by multiplying the sodium and potassium concentrations by the daily urine volume.

Immunoblotting

Semiquantitative immunoblotting was performed to assess the relative expression of NCC protein. Kidneys were quickly removed from anesthetized rats and placed in ice-cold phosphate-buffered saline (RNase-free). All additional steps were performed at 4°C. The capsule was removed, and the cortex was isolated using a tissue slicer (Thomas-Stadie-Riggs; Thomas Scientific, Swedesboro, NJ). The tissue was placed in 2 ml of ice-cold modified RIPA buffer [5 mM Na_2HPO_4, 5 mM NaH_2PO_4, 150 mM NaCl, 2 mM disodium ethylenediaminetetraacetate, 2 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 1% sodium deoxycholate, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 50 mM NaF, 200 mM Na_3VO_4, 0.1% β-mercaptoethanol] with 5 μl/ml protease inhibitors (catalog number 51237; Sigma Chemical Co., St. Louis, MO) and was minced using a tissue grinder (Potter-Elvehjem type; Wheaton Instruments, Millville, NJ). The mixture was then centrifuged for 10 min at 14,000 $\times$ *g*. The supernatant was collected, and the total protein concentration was measured using a Bio-Rad DC kit (Bio-Rad, Richmond, CA). Samples were solubilized with sample buffer (10% glycerol, 5% β-mercaptoethanol, 2.3% sodium dodecyl sulfate, 0.0625 M Tris-HCl).

For immunoblotting, 30 μg of the solubilized proteins were separated by 3 to 8% triacetate polyacrylamide gel electrophoresis and the proteins were electrophoretically transferred to nitrocellulose membranes, as previously described (11). After blocking with Blotto-T (5 g/dl nonfat dry milk and 1 ml/L Tween 20 in phosphate-buffered saline), membranes were probed for 1 h with a previously described, affinity-purified, polyclonal antibody (0.1 μg/ml in Blotto-T) to the amino-terminal domain of the mouse NCC (11). The secondary antibody (0.1 μg/ml) was goat anti-rabbit IgG conjugated to horseradish peroxidase (Zymed Laboratories, South San Francisco, CA). Immunoblots were developed using the ECL Plus kit (RPN2132; Pierce Chemical Co., Rockford, IL) and x-ray film (Kodak, Rochester, NY). As a control, anti-ERp61 antibody was used (StressGen, Victoria, BC, Canada). The band densities of immunoblots were determined using NIH Image version 1.61 software (http://rsb.info.nih.gov/nih-image/).

Northern Blot Analysis

Total RNA was extracted from each renal cortex (Midi kit; Qiagen, Chatsworth, CA). The RNA was dissolved in water, and the concentration was determined by ultraviolet absorbance measurements at 260 nm (DU 640; Beckman Instruments, Palo Alto, CA). The integrity of each sample was verified by electrophoresis on an agarose/formaldehyde gel. Aliquots of 10 μg of total RNA from each cortex sample were separated by 1% agarose/formaldehyde gel electrophoresis and transferred to nylon membranes (Duralon UV; Stratagene, La Jolla, CA). RNA was fixed to the nylon membranes by ultraviolet cross-linking (Stratalinker; Stratagene).

A [α-^{32}P]CTP-labeled full-length riboprobe was generated from the NCC cDNA by random polymerization. As a control gene, a riboprobe was constructed from a 905-bp fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), using the same method.

Membranes were prehybridized for 1 h at 42°C in prehybridization/hybridization solution (Northern Max kit; Ambion, Austin, TX). Membranes were then hybridized for 8 h at 42°C using the same buffer containing 1 ng/ml NCC. Membranes were washed for 15 min with low- and high-stringency solutions (Ambion), and then hybridization for GAPDH was performed using the same method. Membranes were washed again, and hybridization bands were detected by PhosphorImager analysis (Cyclone storage phosphor system; Packard, Meriden, CT). All values were normalized to those for control samples.

Statistical Analyses

Data are expressed as mean $\pm$ SEM. One-way ANOVA and *t* testing were used for statistical analysis (Prism version 2.0, GraphPad Software, Inc., San Diego, CA). $P < 0.05$ was considered statistically significant. The Bonferroni correction was used for multiple comparisons.

Results

Metabolic Effects of Treatment

Animals tolerated each of the three treatment regimens well. Animals in group 1 gained weight throughout the treatment period. As shown in Figure 1, animals in groups 2 and 3 (furosemide and furosemide plus spironolactone) lost weight during the 7-d treatment period. The cumulative weight loss was 28 $\pm$ 3.4 g for group 2 and 31 $\pm$ 4.5 g for group 3.

Figures 2 and 3 present urine volumes and urine composition during the 7-d treatment periods. Urine output was approximately 10 times higher for the furosemide-treated animals

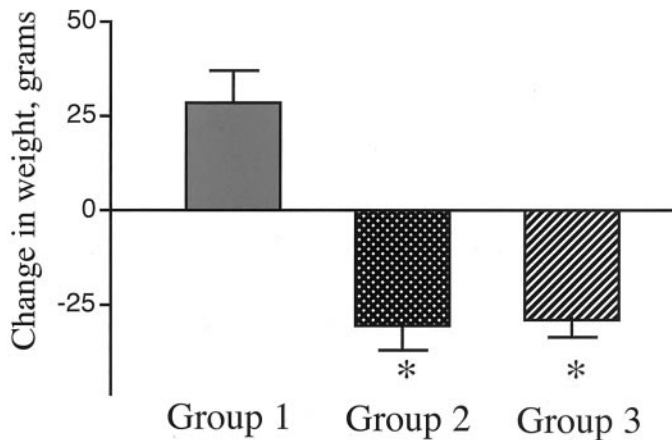

Figure 1. Changes in body weight. Group 1 animals (control) gained weight during the experimental period. Animals in both group 2 (furosemide) and group 3 (furosemide plus spironolactone) lost significant amounts of weight (*, $P < 0.005$, *versus* 0). The weight losses of groups 2 and 3 were not different from each other ($P > 0.05$). Values are means ± SEM for six animals in each group.

(groups 2 and 3), compared with the control animals (Figure 2A). The addition of spironolactone to the furosemide infusion did not further increase urine output, as indicated in a comparison of group 3 with group 2. Urine osmolality decreased during furosemide treatment for groups 2 and 3, compared with the control animals (group 1) (Figure 2B). The urine osmolality was not different between groups 2 and 3. Daily urinary sodium excretion was increased significantly for groups 2 and 3, compared with group 1 (Figure 3A). Rates of urinary sodium excretion were not significantly higher for group 3 than for group 2. Urinary potassium excretion was also increased in the groups treated with furosemide (groups 2 and 3), compared with control animals (group 1) (Figure 3B). Although potassium excretion tended to be lower for group 3 than for group 2, this trend was not significant.

NCC Protein Abundance

Figure 4 demonstrates that furosemide treatment significantly increased the abundance of NCC protein, compared with control values. Also shown in Figure 4 is the effect of spironolactone to reduce NCC abundance when combined with furosemide infusion (group 3). The normalized NCC protein band densities were 100 ± 9% for group 1, 211 ± 16% for group 2, and 140 ± 17% for group 3 ($P < 0.01$ for both group 1 *versus* group 2 and group 2 *versus* group 3). Therefore, spironolactone reduced NCC band density by 66% when infused concomitantly with furosemide. A control protein that is also expressed preferentially by DCT cells was not affected by furosemide or spironolactone treatment (Figure 4).

NCC mRNA Expression

To determine whether the increase in NCC protein abundance was correlated with an increase in NCC mRNA abundance, Northern blotting was performed. Figure 5 presents a representative Northern blot for the three animal groups (placebo-, furosemide-, and furosemide- plus spironolactone-treated groups). Furosemide treatment did not significantly alter NCC mRNA expression, compared with control values. Figure 5 also depicts the NCC/GAPDH expression ratio for each group. Neither treatment significantly affected NCC expression, relative to that of GAPDH. To determine whether a transient increase in NCC mRNA levels caused the increased NCC protein abundance at 7 d, Northern blots were performed with RNA from animals treated for 3 d (group 1, $n = 4$; group 2, $n = 3$). The NCC/GAPDH ratio for the control animals was 0.5 ± 0.12 (arbitrary units), and the ratio for the furosemide-treated animals was 0.5 ± 0.07. These values were not significantly different.

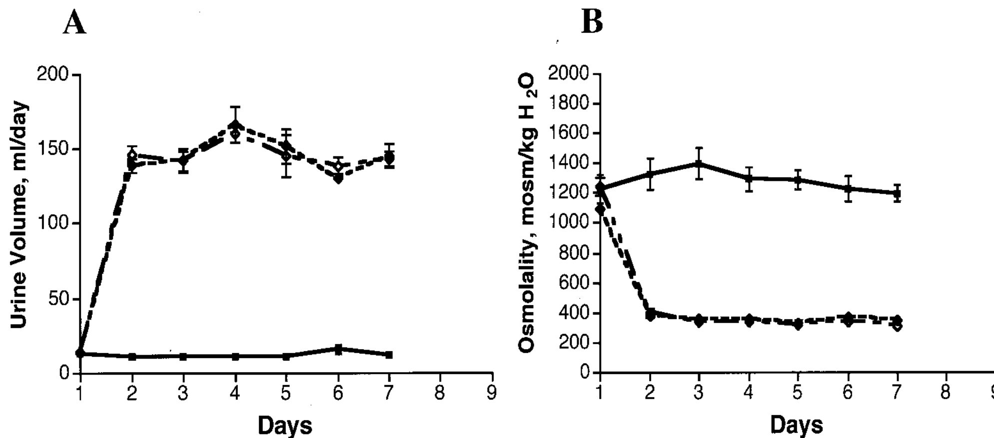

Figure 2. (A) Urine volume increased dramatically for the furosemide-treated animals (group 2, ♦), compared with control animals (group 1, ■; $P < 0.001$). Urine output for the animals treated with furosemide and spironolactone (group 3, ◇) was not different from that observed for the animals treated with furosemide alone ($P > 0.05$). (B) Urine osmolality decreased dramatically for animals treated with furosemide (group 2, ♦), compared with control animals (group 1, ■; $P < 0.001$). The urine osmolality for animals treated with furosemide plus spironolactone (group 3, ◇) was not different from that for group 2 ($P > 0.05$). Values are means ± SEM for six animals in each group.

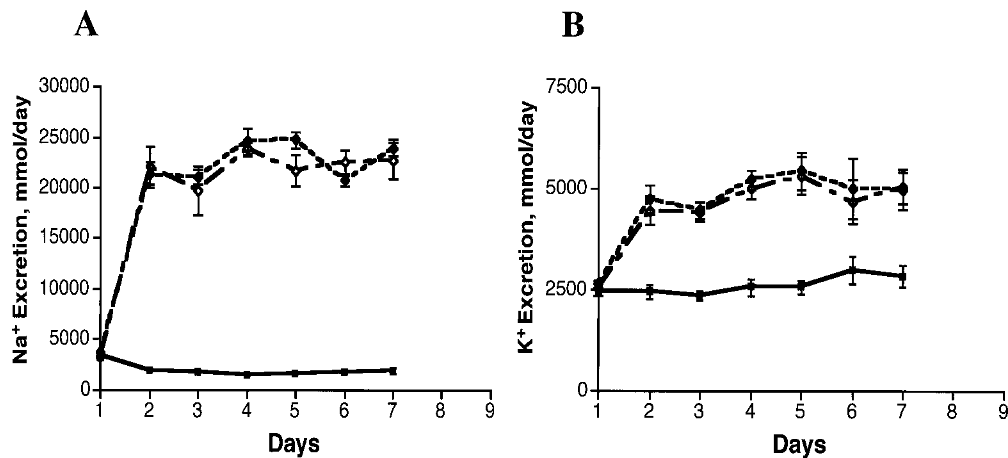

Figure 3. (A) Urinary sodium excretion. Urinary sodium excretion rates were significantly higher for the treatment groups, *i.e.*, group 2 (furosemide; ◆) and group 3 (furosemide plus spironolactone; ◇), compared with control animals (group 1, ■; $P < 0.001$). Sodium excretion rates were not different between group 2 and group 3 ($P > 0.05$). (B) Urinary potassium excretion. Urinary potassium excretion rates were significantly higher for the treatment groups, *i.e.*, group 2 (furosemide; ◆) and group 3 (furosemide plus spironolactone; ◇), compared with control animals (group 1, ■; $P < 0.001$). Although the potassium excretion rate for group 3 tended to be lower than that for group 2, the values were not statistically different ($P > 0.05$). Values are means ± SEM for six animals in each group.

Discussion

The NCC is an electroneutral transport protein expressed at the apical membrane of DCT cells. It is responsible for most of the sodium and chloride reabsorption that occurs in this nephron segment (2). Chronic loop diuretic infusion, together with replacement of NaCl losses, causes DCT cells to become hypertrophic (3,4,9,14); this effect is associated with an increase in NCC activity (4). These effects of chronic loop diuretic infusion are caused in part by increases in distal ion delivery (9), but a role for hormonal factors has not been excluded. The experiments described here tested the hypothesis that chronic loop diuretic infusion increases NCC protein and mRNA expression. Furthermore, they tested the hypothesis that aldosterone contributes importantly to these effects. The results indicate that chronic loop diuretic infusion, with replacement of NaCl losses, increases NCC protein abundance by >100%. The effects of chronic diuretic infusion on NCC protein expression are partially, but not completely, prevented by concomitant spironolactone infusion, indicating an important role for aldosterone in their genesis. The effects do not seem to be caused by an increase in NCC mRNA levels.

These results suggest that several factors may have contributed to the changes in NCC protein abundance during loop diuretic infusion. The first factor is an increase in distal solute delivery. The animals in this study received furosemide in large doses (125 mg/kg per d), leading to a 10-fold increase in daily urine volume. Stanton and Kaissling (5,9) demonstrated that chronic furosemide infusion leads to DCT cell hypertrophy, even when circulating levels of aldosterone, glucocorticoid hormones, and arginine vasopressin are maintained constant. Because the structural effects observed during chronic loop diuretic infusion resemble effects observed in rabbits fed a high-salt diet (15), those authors suggested that the structural and functional effects result directly from changes in solute delivery to the DCT. The results presented here are consistent with a role of luminal solute delivery in the observed increases in NCC protein abundance.

Another potential contributor to the observed effects is a change in total-body electrolyte composition. Furosemide depletes the extracellular fluid volume, which leads to profound hypokalemia. In these experiments, this was reflected by a decrease in body weight (caused at least in part by volume depletion) and an increase in daily urinary sodium and potassium excretion. Each of these changes may have affected ion transport protein expression. However, it seems unlikely that differences in the systemic potassium balance account for the observed effects of furosemide on NCC abundance. Potassium depletion was recently demonstrated to downregulate NCC mRNA expression (16), an effect that is in the opposite direction from that observed during chronic furosemide infusion. Therefore, it seems unlikely that differences in electrolyte balances play a central role in the NCC changes that were observed.

A third factor that may contribute to the observed effects is neurohormonal stimulation. Loop diuretics strongly stimulate renin secretion. Although a portion of this effect is attributable to changes in extracellular fluid volume, the most important component results from direct stimulation of the macula densa mechanism (17). This is confirmed by experiments demonstrating that, even when extracellular fluid volume depletion is prevented by dietary intake, chronic furosemide infusion stimulates the renin/angiotensin/aldosterone system (18). Therefore, chronic furosemide infusion significantly increases serum aldosterone levels, both by reducing extracellular fluid volume and by stimulating renin directly. One indication of an important role of the renin/angiotensin/aldosterone system in the response to loop diuretic administration with NaCl replacement is the effect of angiotensin-converting enzyme inhibition. Beck

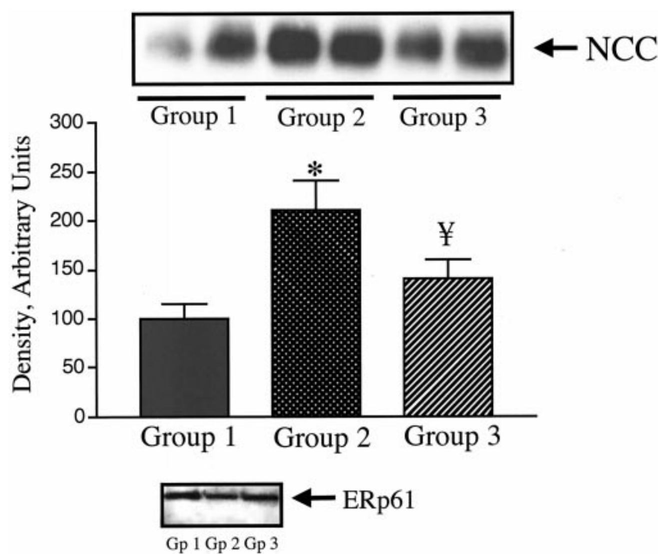

Figure 4. Effects of diuretic treatment on the expression of Na^+/Cl^--cotransporter (NCC) protein, as detected by Western blotting. (Inset at the top) Results from a representative experiment. (Main panel) Means ± SEM of the densitometric values for six animals in each treatment group. Furosemide treatment (group 2) increased protein abundance, compared with control values (group 1) (211 ± 16% of control, $^*P < 0.01$). Treatment with furosemide plus spironolactone (group 3) resulted in NCC expression levels that were intermediate between control values and furosemide treatment values and were significantly less than those for group 2 (140% ± 17 of control; ¥, $P < 0.05$, compared with group 2). (Inset at the bottom) Protein samples from the same treatment groups, probed for another protein expressed predominantly by distal tubule cells, *i.e.*, ERp61, as a control for nonspecific changes (25).

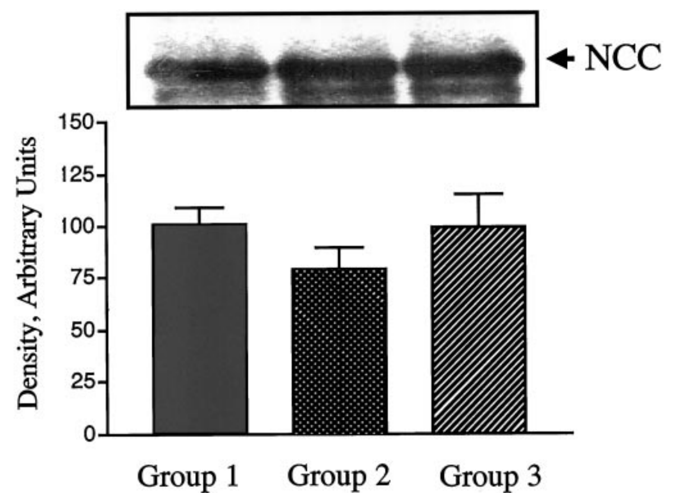

Figure 5. (Inset) Representative Northern blot for the three treatment groups. (Main panel) Means ± SEM of the NCC/glyceraldehyde-3-phosphate dehydrogenase ratios for six animals in each treatment group. Treatment with furosemide or furosemide plus spironolactone had no significant effect on NCC mRNA abundance (group 1 [control], 0.6 ± 0.12; group 2 [furosemide], 0.5 ± 0.05; group 3 [furosemide plus spironolactone], 0.7 ± 0.13; $P > 0.05$).

et al. (13) demonstrated that angiotensin-converting enzyme inhibitors reduced the ability of loop diuretics (with NaCl replacement) to induce epithelial thickening. It has recently become clear that aldosterone regulates NaCl reabsorption in the DCT (2,6,10,12). We previously demonstrated that dietary salt restriction, which increases circulating aldosterone levels, increases the functional activity of the NCC (4). We later confirmed that all of the components of the mineralocorticoid receptor system are expressed by DCT cells, together with the NCC (11), and that aldosterone increased thiazide-sensitive NaCl transport by more than fivefold when infused into adrenalectomized animals (10). Kim *et al.* (12) demonstrated that NCC protein expression was increased in rats who consumed a low-salt diet or were given high doses of aldosterone or fludrocortisone. Therefore, the NCC is an aldosterone-induced protein.

To test whether furosemide-induced increases in circulating levels of mineralocorticoids contribute to the changes in NCC expression, a competitive mineralocorticoid receptor blocker, spironolactone, was used. In pilot studies, a dose of 1.2 mg/dl spironolactone was used. Although this dose has been reported to block >95% of the mineralocorticoid receptors *in vivo* (19), a 20-fold higher dose seemed to offer more complete blockade of furosemide effects and was used for all further experiments. The effects of treatment on body weight, urine volume, urine osmolality, and sodium and potassium excretion were not significantly different between animals treated with both furosemide and spironolactone and animals treated with furosemide alone. Although daily potassium excretion was numerically lower for the group treated with spironolactone, the difference did not reach statistical significance. The absence of a significant effect of spironolactone on potassium excretion could reflect the number of animals tested (a type 2 error), but it could also reflect the fact that much of the potassium that is excreted by these animals is delivered to the DCT (because of the effects of furosemide) and is not secreted along this segment. Furosemide inhibits sodium, potassium, and chloride reabsorption by the thick ascending limb, increasing distal potassium delivery. Furthermore, the animals consume large quantities of a saline drinking solution that contains KCl.

The ability of spironolactone to reduce NCC protein expression levels back toward normal levels indicates that mineralocorticoid hormones contribute importantly to the effects of chronic loop diuretic infusion. Spironolactone does have antiandrogenic effects, which contribute to estrogenic side effects in human patients. Estrogen administration and orchiectomy have both been demonstrated to increase NCC protein abundance (20,21). Because spironolactone reduced NCC protein abundance in the studies presented here, the nonspecific effects of spironolactone cannot have caused this effect. Because addition of spironolactone to a loop diuretic would tend to exacerbate extracellular fluid volume depletion, it seems very unlikely that the effects of spironolactone to reduce NCC protein abundance resulted from increased extracellular fluid volume. Despite the significant ability of spironolactone to shift NCC protein expression toward control levels, its inability

to completely normalize NCC protein abundance indicates either that mineralocorticoid receptor blockade is incomplete or that alternative stimuli, such as those discussed above, contribute to the effects. These experiments cannot differentiate between these two possibilities. Furthermore, these experiments cannot distinguish a permissive role for aldosterone from a direct causative role.

The effects of chronic loop diuretic administration on NCC protein abundance parallel the effects of loop diuretic administration on structural and functional properties of the rat DCT (3–5,8,9). Furthermore, the effects parallel those of high levels of aldosterone on NCC protein abundance (12). Previous data on NCC mRNA expression during chronic loop diuretic infusion have been contradictory, however. We previously demonstrated that loop diuretic infusion increases NCC mRNA expression along the DCT, as detected qualitatively by *in situ* hybridization (7). Those results were recently confirmed in a mouse model, using the same techniques (22). In contrast, Moreno *et al.* (23) reported that chronic furosemide infusion did not affect NCC mRNA expression, as detected by semiquantitative PCR. The results presented here are consistent with those reported by Moreno *et al.* (23) but not with the results determined using *in situ* hybridization. One explanation for our inability to detect an effect of furosemide on NCC mRNA expression in these experiments may be that the signal/noise ratio of Northern blots for the NCC (a low-abundance message in whole kidney cortex) is too low. This would not, however, explain the findings reported by Moreno *et al.* (23), for which specific PCR was used. Another explanation may be that increased NCC mRNA expression occurred at an earlier time point. We demonstrated, however, that NCC mRNA expression was not different at 3 d, *i.e.*, 4 d before the time at which NCC protein abundance was demonstrated to be increased. One possibility is that aldosterone regulates NCC protein abundance independently of NCC mRNA expression, at least in part. Transcription-independent regulation of epithelial sodium channel α (αENaC) synthesis has been clearly demonstrated in A6 cells, where aldosterone increases αENaC protein abundance before any change in αENaC mRNA expression occurs (24).

In conclusion, NCC protein abundance is increased during chronic loop diuretic infusion. This increase in protein abundance may not be caused by an increase in transcription and could be caused by changes in the synthesis, processing, or degradation of the NCC. The furosemide-induced increase in NCC protein abundance is caused in part by aldosterone, because spironolactone (a mineralocorticoid receptor antagonist) partially reversed the upregulation. Other factors, however, may also contribute; a spironolactone-independent increase in NCC protein expression may result from increased luminal solute delivery, as argued previously. These results have implications for clinical practice. Diuretic resistance during loop diuretic administration may have aldosterone-dependent and aldosterone-independent components. Because the aldosterone-dependent component may be blocked or reversed with spironolactone, the combination of spironolactone with a loop diuretic for the treatment of edema may have specific protective effects on the kidney, helping to prevent the development of diuretic resistance.

Acknowledgments

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