Chronic furosemide or hydrochlorothiazide administration increases H⁺-ATPase B1 subunit abundance in rat kidney

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Hydrochlorothiazide (HCTZ) exerts its diuretic action by binding to the Na-K-2Cl cotransporter in the thick ascending limb and blocking ion transport (22). Hydrochlorothiazide (HCTZ) exerts its diuretic action by binding to the Na-K-2Cl cotransporter in the distal convoluted tubule (4). The resultant increase in distal delivery of Na⁺ enhances Na⁺ reabsorption in the connecting tubule and collecting duct, promoting lumen-negative transepithelial voltage and indirectly stimulating proton secretion (11). It appears possible that metabolic alkalosis produced by long-term diuretic administration is in part due to an adaptive increase in H⁺-ATPase in the connecting tubule and collecting duct.

The vacuolar H⁺-ATPase is composed of 13 subunits with several cell- and tissue-specific isoforms (2). The B subunit belongs to the peripheral domain V₁. Two isoforms, B1 and B2, have been identified. The B1 isoform is expressed in all subtypes of intercalated cells of the kidney, whereas the B2 isoform is almost ubiquitously expressed. Mutations in the gene encoding the B1 subunit result in distal renal tubular acidosis (dRTA) in humans who cannot appropriately acidify urine.

We hypothesized that a secondary increase in the distal delivery of Na⁺ may induce a change in H⁺-ATPase downstream from the primary site of the diuretic action. First, we administered either furosemide or HCTZ for 7 days to rats and investigated the effects on the expression of the H⁺-ATPase B1 subunit. Second, we examined whether enhanced distal Na⁺ reabsorption may affect the expression of H⁺-ATPase by coadministering amiloride with furosemide. Third, we investigated which subtypes of intercalated cells were involved in the expression H⁺-ATPase by chronic diuretic administration using antibodies of the H⁺-ATPase B1 subunit and pendrin, an apical Cl⁻/HCO₃⁻ exchanger.

METHODS

Animals and Experimental Protocols

Specific pathogen-free male Sprague-Dawley rats (SLC, Shizuoka, Japan), weighing 170–230 g, were placed in metabolism cages 3 days before the beginning of the study. All of the rats 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. Three different chronic studies were carried out as follows.

Chronic furosemide infusion. Twelve rats were randomly allocated into either the control group or the treatment group. Six 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. Six 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, n = 6) or vehicle (n = 6) for 7 days. These infusions were achieved using the same vehicle solution and osmotic minipumps as described above.

**Chronic furosemide with/without amiloride infusion.** Twelve rats were randomly allocated into three groups: the control (C) group (n = 4), the furosemide (F) group (n = 4), and the furosemide with amiloride (FA) group (n = 4). Rats were infused with 12 mg/day of furosemide (F and FA group) or vehicle (C group) for 7 days through the osmotic minipumps. Amiloride pellets (D-181, Innovative Research of America, Sarasota, FL) or placebo pellets (C-111, Innovative Research of America) were subcutaneously implanted into the rats in the FA group or the C and F groups. For 7 days, 238 μg/day of amiloride were continuously delivered to the rats in the FA group, which was above the rate needed to achieve a drug concentration of 2–5 μM in the lumen of the distal nephron (10).

At the end of each experimental period, the animals were anesthetized by peritoneal injection of ethyl carbamate (33 g melted in 100 ml PBS, 0.5 ml/200 g body wt) before death. All animal procedures were approved by the animal ethics review committee of the Clinical Research Institute of Seoul National University Hospital.

**Physiological measurements.** During the course of the studies, daily body weight and urine volume were measured. A 24-h urine collection was made under saturated thymol and mineral oil. Urine pH was measured with a pH meter (accurate AR15, Fisher Scientific, Pittsburgh, PA), urine electrolytes were measured by an ion-selective method using an automated electrolyte analyzer (EA07S, A&T, Yokohama-shi, Kanagawa, Japan), and urinary ammonium was determined with an ammonia kit (catalog no. 171-C, Sigma, St. Louis, MO). Under anesthesia, blood was collected by aortic puncture. Arterial blood gases were measured at the Department of Laboratory Medicine, Seoul National University Hospital, using a blood-gas analyzer (ABL800 FLEX, Radiometer, Copenhagen, Denmark). Serum aldosterone was measured by radioimmunoassay (SPAC-S Aldosterone kit, Daiichi Pharmaceutical, Tokyo, Japan).

**Preparation of Total RNA**

After death, half of the left kidney from each rat was dissected into small pieces and placed in chilled TRIzol solution (Sigma). The pieces were homogenized with a tissue homogenizer (PowerGun 125, Fisher Scientific), and chloroform (Sigma) was added. After vortexing, 1 ml of the sample was incubated at room temperature for 3 min and centrifuged at 14,000 rpm for 15 min. The transparent portion was incubated at room temperature for 3 min and 75% ethanol was added. Vortexing and centrifugation at 14,000 rpm for 15 min. The isopropyl alcohol was removed after centrifugation. 1 ml nuclease-free water was added. The RNA concentration for 5 min were repeated. After discarding of the ethanol, each pellet of the sample was incubated at room temperature for 10 min, followed by centrifugation at 14,000 rpm for 10 min. The isopropyl alcohol was removed after centrifugation of the presence of RNA pellets at the bottom of each tube, and 75% ethanol was added. Vortexing and centrifugation for 5 min were repeated. After discarding of the ethanol, each pellet was dried and nuclease-free water was added. Vortexing and centrifugation at 14,000 rpm for 10 min. The isopropyl alcohol was removed after centrifugation of the presence of RNA pellets at the bottom of each tube, and 75% ethanol was added. Vortexing and centrifugation for 5 min were repeated. After discarding of the ethanol, each pellet was dried and nuclease-free water was added. Real-time PCR was performed with 1 μl of cDNA and a master mix containing 10 μl of TaqMan 2× PCR Master Mix (Applied Biosystems, Branchburg, NJ), 8 μl nuclease-free water, and 1 μl of TaqMan probe, and primer sets for the H11003 and the reverse primer H11001 and H11002, respectively. The probe contained a 6-carboxy-fluorescenin phosphoramide (FAM dye) label at the 5′-end of the gene, a minor groove binder, and a nonfluorescent quencher at the 3′-end. Each sample was measured in duplicate.

**Antibodies**

To obtain a polyclonal antibody against the H11003-A TPase B1 subunit, the peptide Cys-Pro-Gln-Asp-Thr-Glu-Ala-Asp-Thr-Ala-Leu from the COOH terminus of the bovine kidney isofrom of the ~56-kDa subunit was produced by standard solid-phase peptide synthesis techniques. The peptide was purified by HPLC and was conjugated to maleimide-activated keyhole limpet hemocyanin via covalent linkage to the NH2-terminal cysteine. Two rabbits were immunized with this conjugate using a combination of Freund’s complete and incomplete adjuvants. The rabbits developed ELISA titers >1:32,000 before exsanguination. Preliminary immunoblotting studies revealed that one of these was superior from the perspective of sensitivity and specificity, and all subsequent studies were done with this antiserum (L615). The antiserum was affinity purified using a column on which 2 mg of the same synthetic peptide were immobilized via covalent linkage to maleimide-activated agarose beads (Immunobilization Kit 2, Pierce, Rockford, IL). We also used the rabbit polyclonal anti-pendrin antibody generated against synthetic peptides corresponding to 22 amino acids of the COOH terminus of mouse pendrin conjugated to keyhole limpet hemocyanin, which has been characterized previously (13) and was a gift from Dr. S Nielsen (University of Aarhus, Aarhus C, Denmark).

**Semiquantitative Immunoblotting**

**Preparation of protein samples for immunoblotting.** After death, the right kidneys were rapidly removed and placed in chilled isolation solution containing 250 mM sucrose, 10 mM triethanolamine (Sigma), 1 μg/ml leupeptin (Sigma), and 0.1 mg/ml phenylmethylsulfonyl fluoride (Sigma) titrated to pH 7.6. Next, the kidneys were dissected into the cortex and inner stripe of the outer medulla. Each region was separately homogenized in 10 ml (cortex) or 1 ml (outer medulla) of ice-cold isolation solution using a tissue homogenizer (PowerGun 125, Fisher Scientific). After homogenization, the total protein concentration was measured using the bicinchoninic acid protein assay reagent kit (Sigma) and adjusted to 2 μg/μl with isolation solution. The samples were then stabilized by adding 1 vol 5% Laemmli sample buffer/4 vol sample and heating to 60°C for 15 min.

**Electrophoresis and immunoblotting.** Initially, “loading gels” were done on each sample set. Five micrograms of protein from each sample were loaded into an individual lane and electrophoresed on 12% polyacrylamide-SDS minigels using a Mini PROTEIN III electrophoresis apparatus (Bio-Rad, Hercules, CA) and then stained with Coomassie blue dye (G-250, Bio-Rad; 0.025 percent solution made in 4.5 percent methanol and 1 percent acetic acid). Selected bands from these gels were scanned (GS-700 Imaging Densitometry, Bio-Rad) to determine the density (Molecular Analyst version 1.5,
Bio-Rad), and relative amounts of protein were loaded in each lane. Finally, protein concentrations were “corrected” to reflect these measurements. For immunoblotting, 50 μg of protein from each sample were loaded into each lane and electrophoresed on 10% polyacrylamide-SDS minigels. The proteins were transferred electrophoretically from unstained gels to nitrocellulose membrane (Bio-Rad). After being blocked with 5% skim milk in PBS-T (0.05 mM Na2PO4, 20 mM NaH2PO4, 100 mM NaCl, 0.1% Tween 20, pH 7.5) for 30 min, the membranes were probed overnight at 4°C with the respective primary antibodies. For probing blots, both antibodies were diluted into a solution containing 150 mM NaCl, 50 mM sodium phosphate, 10 mg/ml sodium azide, 50 mg/ml Tween 20, and 0.1 g/ml bovine serum albumin (pH 7.5). The dilutions of primary antibodies used in this study were as follows: anti-H+-ATPase, 1:1,000; anti-pendrin, 1:500. The secondary antibody was goat anti-rabbit IgG conjugated to horseradish peroxidase (31458, Pierce) diluted to 1:3,000. Sites of antibody-antigen reaction were viewed using an enhanced chemiluminescence substrate (ECL RPN 2106, Amersham Pharmacia Biotech, Buckinghamshire, UK) before exposure to X-ray film (Hyperfilm, Amersham Pharmacia Biotech). The secondary antibody was goat anti-rabbit IgG conjugated to horseradish peroxidase (31458, Pierce) diluted to 1:3,000. Sites of antibody-antigen reaction were viewed using an enhanced chemiluminescence substrate (ECL RPN 2106, Amersham Pharmacia Biotech, Buckinghamshire, UK) before exposure to X-ray film (Hyperfilm, Amersham Pharmacia Biotech). Densitometric values were normalized to the mean for the control group in a given experiment, which was defined as 100%, and results are expressed as means ± SE.

Immunohistochemistry

Immunohistochemical staining was performed using 12 rat kidneys in the chronic furosemide with/without amiloride experiment. The other half of the left kidney from each rat was immersed overnight in 4% paraformaldehyde solution at 4°C. After being blocked with 5% skim milk in PBS-T (0.05 mM Na2PO4, 20 mM NaH2PO4, 100 mM NaCl, 0.1% Tween 20, pH 7.5) for 30 min, the membranes were probed overnight at 4°C with the respective primary antibodies. For probing blots, both antibodies were diluted into a solution containing 150 mM NaCl, 50 mM sodium phosphate, 10 mg/ml sodium azide, 50 mg/ml Tween 20, and 0.1 g/ml bovine serum albumin (pH 7.5). The dilutions of primary antibodies used in this study were as follows: anti-H+-ATPase, 1:1,000; anti-pendrin, 1:500. The secondary antibody was goat anti-rabbit IgG conjugated to horseradish peroxidase (31458, Pierce) diluted to 1:3,000. Sites of antibody-antigen reaction were viewed using an enhanced chemiluminescence substrate (ECL RPN 2106, Amersham Pharmacia Biotech, Buckinghamshire, UK) before exposure to X-ray film (Hyperfilm, Amersham Pharmacia Biotech). Densitometric values were normalized to the mean for the control group in a given experiment, which was defined as 100%, and results are expressed as means ± SE.

RESULTS

Characterization of Anti-H+-ATPase B1 Subunit Antibody by Immunoblotting

In this study, we describe a rabbit polyclonal anti-H+-ATPase B1 subunit antibody, L615, raised to a synthetic peptide corresponding to a portion of the COOH terminus of the bovine kidney isoform of the ~56-kDa subunit. Figure 1 shows the immunoblots, each loaded with homogenates from the renal cortex, outer medulla, and inner medulla of a rat. The blots were probed with L615 (left) and with L615 preadsorbed with an excess of the immunizing peptide (right). The antibody recognizes a predominant band of ~56 kDa in both the cortex and medulla, as previously described with another anti-H+-ATPase B1 subunit antibody (17). The band disappeared when the L615 antibody was preadsorbed with an excess of the immunizing peptide.

Responses to Furosemide Infusion

There was no significant difference in body weight between furosemide-infused rats and vehicle-infused controls (Table 1). As expected, urine output was markedly increased by furosemide infusion compared with vehicle-infused controls. From the urine collected at the end of the experiment, sodium and
The expression level of mRNA on whole-kidney isolates from vehicle- and furosemide-infused rats. We found that the expression level of H^+-ATPase B1 subunit mRNA was not changed after 7 days of furosemide infusion (vehicle vs. furosemide, 100 vs. 13%) (Fig. 3). Preliminary 12% SDS-polyacrylamide gels demonstrated a uniform loading among all samples (not shown), ruling out the possibility that the increase in band density in either the cortex or outer medulla could be due to differences in loading.

**Responses to HCTZ Infusion**

Chronic infusion of HCTZ also produced a significant increase in urine volume (Table 2). From the urine collected at the end of the experiment, sodium and chloride excretion was remarkably larger in furosemide-infused rats. Urinary potassium excretion showed a tendency to increase in furosemide-infused rats, but the increase was not statistically significant. Urine pH was significantly decreased in furosemide-infused rats. Urinary NH_4^+ excretion was significantly increased by furosemide infusion. However, urinary NH_4^+ excretion measured on the final day was not significantly increased in HCTZ-infused rats.

Figure 4 shows the results of semiquantitative immunoblotting of the H^+-ATPase B1 subunit from cortical and outer medullary homogenates. As in the furosemide infusion study, the abundance of the H^+-ATPase B1 subunit was increased in both the cortex and outer medulla by HCTZ infusion for 7 days. Normalized band densities for HCTZ vs. vehicle in the cortex were 212 ± 28 vs. 100 ± 24%, respectively. Band densities in the outer medulla were 194 ± 13 vs. 100 ± 13%. Parallel Coomassie blue-stained SDS-polyacrylamide gels demonstrated a uniform loading among all samples (not shown), ruling out the possibility that the increase in band density in either the cortex or outer medulla could be due to differences in loading.

**Responses to Furosemide With/Without Amiloride Infusion**

There was no significant difference in body weight between the rats in the C and the F groups, but the rats in the FA group gained less body weight than those in the C group but not those...
in the F group (Table 3). Urine output was significantly increased in the F and FA groups. No difference in urine output was observed between the rats in the F and the FA groups. Similar to the results from the two previous experiments, urinary sodium and chloride excretion was also remarkably greater in the F and FA groups. The amount of sodium and chloride excretion was not affected by amiloride infusion. Urinary potassium excretion showed a tendency to increase in the F and the FA groups without statistical significance. Although urine pH was significantly decreased by furosemide infusion, coadministration of amiloride did not prevent the reduction of urine pH. Urinary NH₄⁺ excretion was significantly increased in both the F and FA groups. Amiloride-blocking Na⁺ reabsorption in the distal nephron could not inhibit the urinary acidification and the urinary NH₄⁺ excretion induced by furosemide. Although no significant acid/base disturbance was detected by furosemide ± amiloride infusion, the absence of statistical significance is probably related to the fact that these parameters were investigated in very few animals.

Figure 5 shows the results of H⁺-ATPase immunoblotting in cortical homogenates from the rat kidneys of the three different treatment groups. Both furosemide and furosemide plus amiloride infusion significantly increased the abundance of protein, and furosemide infusion alone increased the protein slightly more than furosemide plus amiloride infusion did (C vs. F vs. FA, 100 ± 4 vs. 116 ± 11 vs. 134 ± 14%). We also investigated whether the chronic infusion of furosemide ± amiloride alters the protein abundance of pendrin. Neither furosemide infusion alone nor furosemide plus amiloride infusion changed the abundance of pendrin in rat kidneys (C vs. F vs. FA, 100 ± 4 vs. 116 ± 11 vs. 134 ± 14%) (Fig. 6). Uniform loading was also confirmed from a Coomassie blue-stained loading gel (not shown).

Figure 7 shows H⁺-ATPase and pendrin immunohistochemistry of rat kidneys from the three different treatment groups. The intensity of H⁺-ATPase labeling in the collecting duct did not seem to be different among the three groups (Fig. 7, top). Cells with prominent apical and predominant intracellular localization of H⁺-ATPase are mixed in the collecting duct. Morphometric quantification showed that the percentage of apical H⁺-ATPase-positive cells was not different among the three groups (Fig. 8A). Although the intensity in the immunostaining of pendrin did not change with furosemide ± amiloride infusion, pendrin apical expression was markedly increased in the F and FA groups (Fig. 7, middle). Figure 8B shows morphometric data of pendrin-positive intercalated cells. Double immunolabeling of H⁺-ATPase and pendrin was represented in Fig. 7, bottom. H⁺-ATPase was stained with blue, and pendrin was stained with brown.

The effect of chronic furosemide ± amiloride infusion on H⁺-ATPase B1 subunit mRNA was examined (Fig. 9). Ex-
Expression of H^+-ATPase mRNA was not different among the three groups (C vs. F vs. FA, 100 ± 5 vs. 91 ± 10 vs. 115 ± 10%). Chronic furosemide or amiloride infusion did not affect the expression level of H^+-ATPase in rat kidneys.

**DISCUSSION**

In this study, we demonstrated that enhanced urinary acidification in response to chronic diuretic administration was associated with an increased abundance of the 56-kDa B1 subunit of the vacuolar H^+-ATPase in the connecting tubule and collecting duct. This finding might have contributed to an increase in proton secretion by intercalated cells. Although it might be inferred to cause metabolic alkalosis complicated by long-term diuretic use, we could not demonstrate that metabolic alkalosis was produced by the increased abundance of distal nephron H^+-ATPase itself. In contrast to the experiment using acute amiloride administration (14), chronic coadministration of amiloride did not block this furosemide effect. From our data using antibodies against pendrin and H^+-ATPase, we suggest that the upregulation of H^+-ATPase B1 subunit protein is a result of the changes in all types of intercalated cells secondary to tubular hypertrophy by chronic diuretic administration.

The classic explanation for the increase in urinary acidification with diuretic administration is that sodium absorption may be accelerated by the increase in sodium delivery, result-

![Fig. 5](image1)

![Fig. 6](image2)

**Table 3. Physiological data from chronic furosemide with/without amiloride infusion**

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>F</th>
<th>FA</th>
</tr>
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<tbody>
<tr>
<td><strong>Body weight gain, g</strong></td>
<td>52.0±2.3</td>
<td>42.0±3.6</td>
<td>31.2±5.6*</td>
</tr>
<tr>
<td><strong>Urine</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume, ml/day</td>
<td>13±2</td>
<td>125±15*</td>
<td>111±16*</td>
</tr>
<tr>
<td>pH</td>
<td>8.39±0.05</td>
<td>7.70±0.07</td>
<td>7.58±0.16*</td>
</tr>
<tr>
<td>Na^+, mmol/day</td>
<td>3.0±0.4</td>
<td>20.0±3.2*</td>
<td>18.3±3.1*</td>
</tr>
<tr>
<td>K^+, mmol/day</td>
<td>3.3±0.5</td>
<td>5.0±0.4</td>
<td>5.0±0.4</td>
</tr>
<tr>
<td>Cl^−, mmol/day</td>
<td>3.3±0.6</td>
<td>21.8±3.4*</td>
<td>19.8±3.4*</td>
</tr>
<tr>
<td>NH_4^+, amol/day</td>
<td>15.8±4.9</td>
<td>71.1±11.1*</td>
<td>79.5±21.9*</td>
</tr>
<tr>
<td><strong>Blood</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>pH</td>
<td>7.36±0.02</td>
<td>7.45±0.01</td>
<td>7.44±0.03</td>
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<tr>
<td>Pco_2, mmHg</td>
<td>43.2±2.5</td>
<td>33.3±0.5</td>
<td>38.4±4.1</td>
</tr>
<tr>
<td>HCO_3^−, mmol/l</td>
<td>23.6±1.0</td>
<td>22.9±0.4</td>
<td>25.8±0.7</td>
</tr>
</tbody>
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Values are means ± SE. C, controls; F, furosemide; FA, furosemide + amiloride. *P < 0.05 vs. C.
ing in an increase in lumen-negative transepithelial potential difference (5, 7). The increase in transepithelial voltage is believed to accelerate proton secretion by altering the electrochemical driving force against which the H\textsuperscript{+}-H\textsubscript{11001}-ATPase must pump (11). An intercalated cell-specific H\textsuperscript{+}-H\textsubscript{11001}-ATPase B1 subunit is essential for renal proton secretion, which was demonstrated in the recent experiment where the normal urinary acidification induced by a lumen-negative potential in response to furosemide infusion was abolished in mice deficient for the H\textsuperscript{+}-ATPase B1 subunit (8). Kovacikova et al. (14) recently concluded that the H\textsuperscript{+}-ATPase B1 subunit was necessary for furosemide-induced urinary acidification and that the connecting tubule was a major segment in electrogenic urinary acidification. However, there was a recent argument against this explanation that the changes in transtubular voltage by the action of the epithelial sodium channel (ENaC) were minor compared with the ability of the pump to be active against a larger voltage gradient (28). The present results provide an additional mechanism, that diuretic administration may enhance proton secretion by increasing the abundance of H\textsuperscript{+}-ATPase B1 subunit protein. The change in the abundance of this protein after diuretic administration is unique, because the B1 protein level was not increased after oral acid loading (7). To test whether enhanced sodium reabsorption was involved in this phenomenon, we simultaneously administered the ENaC inhibitor amiloride with furosemide. Chronic coadministration of amiloride with furosemide could neither definitely inhibit furosemide-induced urinary acidification nor decrease the upregulated H\textsuperscript{+}-ATPase expression. In other words, amiloride’s ENaC blocking effect had little influence on chronic furo-
Furosemide treatment could increase H^+ secretion in parallel with active H^+ secretion (9) because of decreased urine pH and increased urinary flow rate, which could reduce collecting duct NH3 concentration and enhance the gradient for NH3 diffusion across the collecting duct (5). Amiloride infusion did not affect urinary NH3 excretion in either our chronic study or the acute study of Kovacikova et al. (14). Although a net acid excretion measurement could have given additional information, we did not measure net acid excretion or titratable acidity because the urine pH in our study was too high to titrate to pH 7.4.

Fig. 8. Subcellular redistribution of H^+-ATPase and pendrin by the infusion of furosemide with/without amiloride. Morphometry reveals that the apical localization of H^+-ATPase is not different among the 3 treatment groups (A). However, intercalated cells with apical pendrin immunostaining are more frequent in the F and FA groups (B). *P < 0.05.

In the experiment of acute furosemide and subsequent amiloride application, the stimulation of urinary acidification was abolished by amiloride. The fractional excretion of sodium and chloride was increased in the furosemide plus amiloride treatment group compared with the control and furosemide treatment groups (14). However, in our experiment, coadministration of amiloride did not make any impact on the changes in physiological parameters caused by furosemide infusion. If we had compared the urinary data between the F and the FA groups after several hours of starting the infusion, there might have been some differences in the physiological data between these two groups. Chronic administration of furosemide and amiloride for 7 days might have blurred the difference because the animals were adapted to those stimuli and remained in a steady state. Another possible explanation is the difference in the dose of amiloride. We administered a total of 238 μg of amiloride/day, which was nearly equal to the dose of 30 nmol/h from previous study (10). Kovacikova et al. (14) administered a bolus of 2 μg/g body wt, almost five times the daily dose in our study. Thus the dose of amiloride in our study may not have been large enough to make any physiological difference. Urinary NH3 excretion was increased by furosemide and furosemide plus amiloride, but not by HCTZ. These results are compatible with those in a previous study (12) showing that urinary acidification is stimulated by loop diuretics. The increase in NH3 excretion might be due in part to enhanced passive diffusion of NH3 in parallel with active H^+ secretion (9) because of decreased urine pH and increased urinary flow rate, which could reduce collecting duct NH3 concentration and enhance the gradient for NH3 diffusion across the collecting duct (5). Amiloride infusion did not affect urinary NH3 excretion in either our chronic study or the acute study of Kovacikova et al. (14). Although a net acid excretion measurement could have given additional information, we did not measure net acid excretion or titratable acidity because the urine pH in our study was too high to titrate to pH 7.4.

Fig. 9. Effects of furosemide with/without amiloride infusion on the expression of H^+-ATPase mRNA. H^+-ATPase mRNA expression was determined by real-time quantitative RT-PCR using total renal RNA from Sprague-Dawley rats in the 3 groups. A 7-day infusion of furosemide or furosemide with amiloride did not alter the expression of H^+-ATPase mRNA.

Pendrin differentiates type B and non-A non-B intercalated cells from type A cells (13, 21, 26). Pendrin has been known to be regulated by aldosterone and chloride (19, 23, 24, 27, 25). In our study, chronic furosemide or furosemide plus amiloride administration did not affect the total abundance of pendrin, but those treatments redistributed pendrin from the cytoplasm to the apical membrane. Because the main mechanism of pendrin regulation is subcellular redistribution, we think that non-A intercalated cells were activated. There are a few studies reporting the changes in pendrin by the administration of diuretics (18, 19, 23). Pendrin protein expression and the number of pendrin-positive cells were increased in NaCl co-transporter (NCC) knockout mice, which had a chronic defect in NaCl transport (23). The reason why no change in pendrin protein abundance was observed in our study could be interpreted as the differences between the animals studied. Even though the rats in our study had received the diuretics chronically, they might have had definite differences from the NCC.
knockout mice, which had a lifelong adaptation to the loss of NaCl. To supplement NaCl or not during chronic furosemide administration could make a difference in the level of pendrin protein expression (19). Increased pendrin protein expression by furosemide in immunohistochemistry, as demonstrated from the experiment done by Pech et al. (18), needs to be confirmed quantitatively by immunoblotting.

Eiam-Ong et al. (6) showed that the increase in collecting duct H^+ATPase activity was associated with an increase in plasma aldosterone level in furosemide-infused rats. Also, other studies have demonstrated that furosemide’s effects on increasing urinary acidification can be observed when changes in plasma mineralocorticoid level, extracellular fluid volume, and plasma potassium concentration are prevented (1, 3, 20). The direct influence of furosemide on distal acidification is an appropriate explanation in our study because there was no change in aldosterone level. Levine et al. (15) showed that angiotensin II stimulated urinary acidification and an adaptive increase in H^+ATPase expression in unilaterally nephrectomized rats. Therefore, we cannot rule out the possibility that an increased angiotensin II level might influence H^+ATPase expression and urinary acidification in this experiment.

In summary, chronic diuretic administration enhanced distal acidification and increased the protein abundance of the B1 subunit of vacuolar H^+ATPase, an effect which was not inhibited by amiloride. This upregulation of H^+ATPase may be a result of the changes in all types of intercalated cells secondary to the tubular hypertrophy by chronic diuretic administration. The signals underlying these chronic adaptations remain to be elucidated.

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GRANTS

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