Upregulation of the Secretory-Type Na\(^+/K^+\)/2Cl\(^-\)-cotransporter in the Kidney by Metabolic Acidosis and Dehydration in Rats

MIKA IKEBE, HIROSHI NONOGUCHI, YUSHI NAKAYAMA, YUKA TASHIMA, and KIMIO TOMITA

Third Department of Internal Medicine, Kumamoto University School of Medicine, Kumamoto, Japan.

Abstract. The functional role and mechanisms of regulation of the Na\(^+/K^+\)/2Cl\(^-\)-cotransporter NKCC1 in the kidney have not yet been clarified. NKCC1 mRNA and protein expression in control rats, rats with dehydration (2 d), and rats with metabolic acidosis (NH\(_4\)Cl in the food for 6 to 7 d) was examined using reverse transcription-PCR and Western blotting. In contrast to the abundant NKCC1 mRNA expression in the terminal inner medullary collecting ducts in mice, expression was found to be most abundant in the outer medullary collecting ducts (OMCD) in rats. Dehydration and metabolic acidosis increased NKCC1 mRNA expression three- to fivefold not only in the OMCD but also in the cortical collecting ducts and inner medullary collecting ducts. Dehydration and metabolic acidosis increased NKCC1 protein expression two-fold in the membrane fraction from the outer medulla. NKCC1 protein expression was observed not in the microdissected medullary thick ascending limbs but in the OMCD, and it was stimulated twofold by dehydration and metabolic acidosis. Incubation of OMCD in low-pH medium increased NKCC1 mRNA expression. In summary, NKCC1 mRNA and protein expression is upregulated with dehydration and metabolic acidosis. NKCC1 may play an important role in adaptation to these physiologic conditions. Low pH and possibly hypertonicity stimulate NKCC1 mRNA expression in OMCD.

Electroneutral Na\(^+/K^+\)/2Cl\(^-\)-cotransporters play an important role not only in sodium chloride absorption but also in sodium chloride secretion for the maintenance of body fluid homeostasis (1). Two types of bumetanide-sensitive Na\(^+/K^+\)/2Cl\(^-\)-cotransporters and thiazide-sensitive Na\(^+/Cl^-\)-cotransporters have been cloned to date (2–6). The cotransporters belong to the family of integral membrane transport proteins that mediate electrically neutral transport of sodium, potassium, and chloride across the membrane. The two types of bumetanide-sensitive Na\(^+/K^+\)/2Cl\(^-\)-cotransporters are referred to as NKCC1 (BSC2) and NKCC2 (BSC1). The secretory type, NKCC1, is present in various secretory epithelia, whereas the absorptive type, NKCC2, is located only in the kidney. Immunohistochemical studies have demonstrated the presence of NKCC2 proteins in medullary thick ascending limbs (MAL) and cortical thick ascending limbs (CAL) (7,8). NKCC2 mRNA expression in MAL and CAL is stimulated by sodium loading (9). Because NH\(_4^+\) is absorbed in MAL and CAL by substituting for K\(^+\) via NKCC2 (10–12), NKCC2 expression has been thought to be stimulated by metabolic acidosis. In fact, NH\(_4^+\) absorption in rat MAL is increased during metabolic acidosis (13). Dehydration and metabolic acidosis, however, have not been found to affect NKCC2 mRNA expression in these segments (14,15), which suggests that the increase in NH\(_4^+\) absorption may not occur through the changes in NKCC2 protein expression.

NKCC1 has been cloned from mouse terminal inner medullary collecting duct (IMCD) cells, as well as the shark rectal gland and human colon (2–4). Northern blot analyses have demonstrated the presence of NKCC1 mRNA not only in the kidney but also in many secretory epithelia, in sites such as the salivary gland, stomach, jejunum, colon, lung, and heart. Immunohistochemical studies in the kidney have demonstrated the presence of NKCC1 proteins in terminal IMCD in mice but not in terminal IMCD in rats (16,17); the NKCC1 protein has been found to be present in the basolateral membrane of intercalated cells in the outer medullary collecting duct (OMCD) in rats. The OMCD is important not only in acid excretion but also in sodium absorption and secretion (18,19). One role of NKCC1 in rat OMCD has been suggested to be ammonium excretion (17). One of the splice variants of NKCC2, i.e., NKCC2F, has been reported to be located also in the OMCD (14). The direction of sodium movement is different for NKCC1 and -2. Recent reports have revealed that mice lacking NKCC1 exhibit signs of deafness and imbalance (20,21). The functional role of NKCC1 in rat OMCD is still unclear, and it is not known whether NKCC1 expression is regulated by pathophysiologic changes such as dehydration and metabolic acidosis. Therefore, we investigated, using competitive PCR and Western blot analysis, whether NKCC1 ex-
expression is regulated by pathophysiologic changes in body fluid homeostasis.

Materials and Methods

Materials

The cDNA synthesis kit and PCR master kit were obtained from Boehringer Mannheim (Mannheim, Germany). Enhanced chemiluminescence Western blotting detection reagents were purchased from Amersham Pharmacia Biotec (Buckinghamshire, UK). Vanadyl ribonucleoside complex (VRC) was supplied by Life Technologies BRL (Gaithersburg, MD). Agarose was obtained from SeaKem (Rockland, ME). Bacto agar was purchased from Difco Laboratories (Detroit, MI).

Preparation of Rats

The appropriate conditions for inducing metabolic acidosis were first examined. Because conventional 0.28 M NH₄Cl solution is hypertonic, rats not only experience acidosis but also become dehydrated. Therefore, isotonic 0.14 M NH₄Cl solution was administered as drinking water. NH₄Cl was then provided in the food. Several chows for 6 to 7 d (rat chow, 8 g; NH₄Cl, 0.2 g; Bacto agar, 0.25 g; water, 10 ml/100 g body wt per d; X = 0.1, 0.2, 0.25, or 0.5) were administered for 4 d to the acidosis group, for determination of the appropriate doses of NH₄Cl. Arterial blood gas values, plasma osmolality, and plasma vasopressin concentrations were measured.

Using the results from the aforementioned experiments, dehydration and chronic metabolic acidosis were induced in male Sprague-Dawley rats. Dehydration was induced by water deprivation for 2 d. Metabolic acidosis was induced by adding NH₄Cl to standard rat chow for 6 to 7 d (rat chow, 8 g; NH₄Cl, 0.2 g; Bacto agar, 0.25 g; water, 10 ml/100 g body wt per d). Control rats for acidosis received the same diet as did the acidosis group except for the addition of NH₄Cl. Control rats and rats with acidosis were given free access to tap water. After anesthesia with sodium pentobarbital, arterial blood was drawn from the abdominal aorta. Blood pH, PaCO₂, PaO₂, and HCO₃⁻ concentrations were measured using an automatic blood gas analyzer (ABL 510; Radiometer, Copenhagen, Denmark). Plasma levels of vasopressin were measured by RIA (SRL Laboratories, Tokyo, Japan). Plasma osmolality was measured using an osmometer (Fiske, Norwood, MA).

Microdissection of Glomeruli and Nephron Segments

After perfusion of the left kidney with solution A containing 0.1% collagenase and 0.1% bovine serum albumin, microdissection of glomeruli and nephron segments was performed in the presence of 10 mM VRC, as described previously (22,23). The microdissected nephron segments were as follows: glomeruli, proximal convoluted tubules, proximal straight tubules, MAL, CAL, cortical collecting ducts (CCD), OMCD, and IMCD. After the VRC was washed out of the tubules with solution A, five glomeruli and 2-mm-long nephron segments were transferred into Eppendorf tubes, to which 10 ml of solution A2 was added. Solution A had the following composition: 130 mM NaCl, 5 mM KCl, 1 mM Na₂HPO₄, 1 mM MgSO₄, 1 mM calcium lactate, 2 mM sodium acetate, 5.5 mM glucose, and 10 mM Hepes (pH 7.4 with NaOH). Solution A2 had the same composition as solution A except that 5 U/μl RNase inhibitor (Boehringer Mannheim) and 1 M tri-dithiothreitol (DTT; Sigma) were added.

Incubation Study

To elucidate whether low pH stimulates NKCC1 mRNA expression, microdissected OMCD (2 mm) were incubated in 100 μl of isotonic medium at 37°C. The time course of the effect of low pH on NKCC1 mRNA expression was investigated first. Microdissected OMCD were incubated for 0, 5, 15, 30, 60, or 90 min at 37°C in medium with normal or low pH (pH 7.4 and 6.7, respectively). OMCD were then incubated at pH 7.4, 7.1, or 6.7 for 90 min at 37°C. The pH of the medium was adjusted by adding HCl to solution A.

Reverse Transcription-Competitive PCR

Reverse transcription (RT)-competitive PCR was performed using a cDNA synthesis kit and PCR master kit, as described previously (22,23). Each sample was centrifuged at 15,000 rpm for 5 min at 4°C. After the supernatant was discarded, 3.5 μl of 2% Triton X-100 solution with RNase inhibitor and DTT was added. Then, 4.4 μl of a RT mixture containing random primers was added. RT was performed by incubating the samples at 42°C for 60 min. After RT was stopped by heating the sample at 90°C for 5 min, the samples were subjected to PCR.

Specific primers for NKCC1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed. NKCC1 sense and antisense primers were defined by bases 2494 to 2511 (5'-GGTTCTC-CAAACCTACGGG-3') and bases 3040 to 3059 (5'-GTCTTGGCATCCTTTCTCC-3'), respectively, of the mouse NKCC1 cDNA sequence (3). These correspond to bases 2486 to 2505 and 3032 to 3051, respectively, of the rat sequence (24). The amplified cDNA was predicted to be 566 bp in length. The PCR product was sequenced. GAPDH sense and antisense primers were defined by bases 506 to 525 (5'-TCCCTCTAAGATTTGTGCAGCA-3') and bases 794 to 813 (5'-AGATCCACACAGGATACATT-3'), respectively (25). The predicted length of the amplified cDNA was 308 bp. NKCC1 mRNA expression was quantified using competitive PCR. The DNA competitor for the competitive PCR was synthesized using overlap-extension PCR, as described previously (22,23,26,27). The inner antisense and inner sense primers were defined by bases 2682 to 2701 (5'-CCACATCTCCTATCTGTCTTGGCGAGACGATCG- GTGA-3') and bases 2790 to 2809 (5'-TCACCGAGATGACTTGC- GQGGAAAACGATAGGAGGATGTGG-3'), respectively. The inner sense and inner antisense primers were made to be complementary by adding complementary sequences to each 5'-end (the complementary sequences added to each 5'-end are underlined). The first PCR was performed with the combinations of the outer sense and inner antisense primers and the outer antisense and inner sense primers. Two PCR products (208 and 270 bp) were combined in the second PCR. The final PCR product, the DNA competitor, was electrophoresed and purified using a nucleic acids extraction kit, NucleoSpin (Macherey-Nagel, Duren, Germany). The size of the DNA competitor was 478 bp. A series of dilutions of the competitor and NKCC1 cDNA were used to establish the standard curve.

Another DNA competitor was produced using a competitive DNA construction kit (Takara, Shiga, Japan), according to the instructions provided by the manufacturer. Sense and antisense primers for NKCC1 mRNA were used to produce the competitor. The size of the DNA competitor was 456 bp.

GAPDH mRNA was amplified using the usual PCR method, and NKCC1 mRNA was amplified using competitive PCR. The DNA competitor (1 to 20 fg) was added to the NKCC1 samples. The annealing temperatures for NKCC1 and GAPDH were 60°C and 62°C, respectively. The PCR cycle numbers for NKCC1 and GAPDH were 30 and 28 cycles, respectively.

Ethidium Bromide Staining and Southern Blotting

After PCR, the PCR product was precipitated with ethanol and electrophoresed in a 2% agarose gel with Tris-ethylenediaminetetra-
acetic (EDTA) buffer. The PCR products were observed by ethidium bromide staining. The intensity of the bands in the NKCC1 samples was quantitated using a densitometer (Atto, Tokyo, Japan) for competitive PCR assays. The NKCC1 cDNA/competitor ratio was corrected for the differences in molecular weight. The Tris-EDTA buffer had the following composition: 40 mM Tris, 1 mM EDTA (pH 8.3 with acetic acid).

The gel of the GAPDH sample was then denatured and neutralized. The PCR products were transferred onto a nylon membrane with 20× SSC buffer (3 M NaCl, 0.3 M sodium citrate) and left overnight. The membrane was baked in an ultraviolet baking system and stored until Southern hybridization. The nonisotopic digitigoxigenin (DIG)-nucleotide detection system was used for Southern hybridization and DNA detection. A specific probe for GAPDH was produced with a PCR DIG probe synthesis kit, using each cDNA and specific primers, as described in the instruction manual. Observation of DNA was performed using a DIG luminescence detection kit.

Western Blotting

Western blotting was performed using membrane fractions from the cortex, outer medulla, and inner medulla and microdissected OMCD and MAL, as described previously (22,23,28). In brief, the kidney was removed and cut into blocks of the cortex, outer medulla, and inner medulla. Each part was cut into small pieces with a razor blade and homogenized with glass homogenizer. The homogenate was centrifuged at 7600 rpm for 15 min at 4°C. The supernatant was then centrifuged at 15,000 rpm for 30 min at 4°C. The pellet was dissolved in lysis buffer, and the protein content was measured using the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL). Microdissected OMCD (20-mm long) and MAL (20-mm long) were transferred to Eppendorf tubes, to which lysis buffer was added. Samples (30 μg of the membrane fractions and microdissected OMCD and MAL) were mixed with 2× sample buffer and then subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. After SDS-polyacrylamide gel electrophoresis, the proteins were transferred to a nylon membrane (Immobilon-P; Millipore, Bedford, MA). After blocking with Tris-buffered saline with Tween 20 and 5% milk, the membrane was incubated overnight with the primary antibody against NKCC1 protein was a kind gift from Dr. R. James Turner, National Institutes of Health (Bethesda, MD) (24). The antibody was raised against a 6× His fusion protein corresponding to amino acids 750 to 1203 of rat NKCC1. The membrane was then incubated with horseradish peroxidase-linked anti-rabbit IgG F(ab')2, for 1 h at room temperature. Protein expression was detected with enhanced chemiluminescence Western blotting detection reagents (Amersham). Protein expression was measured with a densitometer (Atto). The composition of the lysis buffer was 0.5 mM Na2VO4, 1 mM EDTA, 5 mM ethyleneglycol bis(β-aminoethyl ether)-N,N',N',N'-tetraacetate, 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leucine, 5 mM β-glycerophosphate, and 20 mM Hepes (pH 7.4). The composition of the 2× sample buffer was 20% glycerol, 4.6% SDS, 10% β-mercaptoethanol, 130 mM DTT, 0.01% bromphenol blue, and 130 mM Tris-HCl (pH 6.8). Tris-buffered saline contained 20 mM Tris and 137 mM NaCl.

Statistical Analyses

Results are expressed as mean ± SEM. Statistical analyses were performed using the t test for two groups and ANOVA or Kruskal-Wallis analysis followed by Dunnett’s multiple-comparison test for more than three groups, as appropriate. P < 0.05 was considered significant.

Results

Physiologic Parameters for Rats

The effect of isotonic 0.14 M NH4Cl on plasma bicarbonate concentrations was first examined. However, the reduction in plasma bicarbonate concentrations was not enough (24.5 ± 0.6 and 22.1 ± 1.1 mEq/liter HCO3− in control and NH4Cl-treated rats, respectively; n = 5). The effect of NH4Cl in the food was then examined. Table 1 summarizes the effects of several doses of NH4Cl in the food on several parameters in control rats, dehydrated rats, and rats with metabolic acidosis. Dehydration caused increases in plasma osmolality and arginine vasopressin levels. In contrast, NH4Cl in the food did not increase plasma osmolality or arginine vasopressin levels. Decreases in plasma pH and bicarbonate concentrations were observed at all tested doses. NH4Cl in the food dose-dependently decreased plasma bicarbonate concentrations. However, 0.5 g of NH4Cl/8 g of food caused severe metabolic acidosis and a loss of body weight. On the basis of these results from a variety of in vivo models, 0.2 g of NH4Cl/8 g of standard chow was used to induce metabolic acidosis in the following experiments. These

<table>
<thead>
<tr>
<th>Table 1. Effects of several doses of NH4Cl on parameters for control rats, rats with metabolic acidosis, and dehydrated rats (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH4Cl Treatment (g/8 g of chow per 100 g of body wt per d)</td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>Body weight (g)</strong></td>
</tr>
<tr>
<td><strong>pH</strong></td>
</tr>
<tr>
<td><strong>HCO3− concentration (mEq/liter)</strong></td>
</tr>
<tr>
<td><strong>Osmolality (mosmol/kg H2O)</strong></td>
</tr>
<tr>
<td><strong>Vasopressin concentration (pg/ml)</strong></td>
</tr>
</tbody>
</table>

\(^a\) n = 5 to 8. NH4Cl in the food for 4 d induced metabolic acidosis without dehydration.

\(^b\) P < 0.05 versus control (no NH4Cl) by ANOVA and Dunnett’s multiple-comparison test.
data demonstrated that metabolic acidosis could be induced by our new method without dehydration.

**NKCC1 mRNA Distribution along the Nephron in Mice and Rats**

NKCC1 mRNA distribution along the nephron was compared between mice and rats (Figure 1). NKCC1 mRNA expression was most abundant in the glomeruli and IMCD in mice. Low expression was observed in other nephron segments, such as the proximal convoluted tubules, proximal straight tubules, MAL, CAL, CCD, OMCD, and IMCD. In contrast, NKCC1 mRNA expression was abundant in the collecting ducts, especially the OMCD, in rats. Low expression was observed in the glomeruli, MAL, and CAL. Competitive PCR revealed that NKCC1 mRNA expression in the OMCD was 5 times higher than that in the CCD and 2 times higher than that in the IMCD (Figures 2 and 3).

**Effects of Dehydration and Metabolic Acidosis on Rat NKCC1 mRNA Expression in the Collecting Duct**

NKCC1 mRNA expression was compared in control rats, dehydrated rats, and rats with acidosis. Competitive PCR was used in these experiments. Dehydration significantly increased NKCC1 mRNA expression in the CCD, OMCD, and IMCD (Figure 2). Chronic metabolic acidosis also caused increases in NKCC1 mRNA expression in the CCD, OMCD, and IMCD (Figure 3). Expression was most abundant in the OMCD in both dehydrated rats and rats with metabolic acidosis. There was no difference in GAPDH mRNA expression in the three segments in dehydrated rats, rats with acidosis, and control rats (data not shown). These data show that NKCC1 mRNA expression is regulated under such pathophysiologic conditions.

**Effects of Low Medium pH on Rat NKCC1 mRNA Expression In Vitro**

Time-course experiments revealed that NKCC1 mRNA expression was stimulated by 30- to 90-min incubations in low-pH medium (Figure 4). Although incubation of OMCD at low pH for 5 min decreased NKCC1 mRNA expression, the initial decrease in NKCC1 mRNA expression produced by low pH could be attributable to mRNA degradation. It is not known whether this degradation is caused by the activation of RNase. On the basis of these findings, OMCD were incubated at pH 7.4, 7.1, and 6.7 for 90 min. Incubation of OMCD in pH 6.7 medium significantly stimulated NKCC1 mRNA expression in vitro by 62%, compared with incubation at pH 7.4. The increase in expression at pH 7.1 was not statistically significant (6.8 ± 0.6, 7.9 ± 1.0, and 11.0 ± 1.4 fg/mm at pH 7.4, 7.1, and 6.7, respectively; P < 0.05 for pH 6.7 versus pH 7.4) (Figure 5).

**Rat NKCC1 Protein Expression in Dehydrated Rats, Rats with Metabolic Acidosis, and Control Rats**

The antibody recognized a single broad band at 170 kD, which was compatible with the predicted size of the NKCC1...
protein. The membrane fraction from the inner medulla exhibited higher levels of expression of NKCC1 protein than did the cortex or the outer medulla (Figure 6). Dehydration and acidosis significantly increased NKCC1 protein expression in the membrane fraction from the outer medulla, by 122 ± 35 and 95 ± 29%, respectively. This increase was not observed in other parts of the kidney.

NKCC1 protein expression was observed in microdissected OMCD but not in MAL, confirming that this antibody is specific for NKCC1 (Figure 7). Dehydration and chronic metabolic acidosis significantly increased expression, by 113 ± 23 and 86 ± 30%, respectively.

Discussion
Our data showed that (1) NKCC1 mRNA distributions are different in mice and rats, with expression being most abundant in IMCD in mice but in OMCD in rats, (2) NKCC1 mRNA expression in the collecting ducts is stimulated by dehydration and chronic metabolic acidosis, (3) NKCC1 mRNA expression is stimulated by low medium pH in vitro, (4) NKCC1 protein expression can be observed in microdissected OMCD but not in MAL, and (5) NKCC1 protein expression is stimulated by dehydration and metabolic acidosis. Considering the findings that NKCC2 mRNA expression and protein expression in MAL and CAL are not upregulated by these physiologic changes related to body fluid homeostasis (9,14,15), it can be concluded that both NKCC1 and NKCC2 are regulated with changes in water and electrolyte balances, e.g., via vasopressin, although the mechanisms of these effects likely differ for these transporters.

NKCC2 has been considered to be more important than NKCC1 for sodium absorption and secretion. The distribution of NKCC2 is only in the kidney. Immunohistochemical studies have demonstrated the presence of NKCC2 in the luminal membrane of MAL and CAL, where the capacity for NaCl
absorption is very high and is sensitive to arginine vasopressin. NKCC2 protein levels in MAL are not upregulated with water restriction or vasopressin treatment, although vasopressin increases NKCC2-mediated sodium reabsorption (9,29). Therefore, vasopressin seems to regulate NKCC2 through another mechanism. Furosemide and bumetanide are known to cause potent diuresis by inhibiting NKCC2. In contrast, NKCC1 is located in various secretory epithelia, including the OMCD of rat kidney. Although OMCD have a lower sodium-absorbing capacity than do MAL and CAL (19), the epithelial sodium channel is known to be activated by vasopressin and aldosterone (30). OMCD also have a high capacity for acid excretion (18). In this study, chronic metabolic acidosis increased NKCC1 mRNA expression in OMCD. Furthermore, incubation of OMCD in low-pH medium for >30 min stimulated NKCC1 mRNA expression in vitro, suggesting that low pH is one of the stimulators of NKCC1 mRNA expression. The initial decrease in NKCC1 mRNA expression could be attributable to the increased degradation of mRNA. RNase activation may play some role. Wall et al. (31) reported that increased extracellular pH stimulated bumetanide-sensitive rubidium uptake in mouse IMCD cells, which is a change in the opposite direction, compared with our results. Their study examined very acute effects of the changes in extracellular pH on NKCC1 activity. The stimulation of NKCC1 activity was probably caused without changes in NKCC1 mRNA or protein expression. Our study focused on chronic effects of extracellular pH. Initial decreases in NKCC1 mRNA expression with low pH may reflect acute inhibitory effects of low pH on NKCC1 mRNA expression. Therefore, it might be reasonable to assume that low extracellular pH inhibits NKCC1 mRNA expression in the acute phase but stimulates it in the chronic phase.

Net acid excretion is the sum of ammonium and titratable acid excretion if there is no bicarbonate-wasting. Ammonia is primarily produced in proximal tubules by phosphate-dependent glutaminase and accumulates in the medullary interstitium (11). The ammonium ion (NH₄⁺) is absorbed via NKCC2 in MAL and CAL by substituting for K⁺ (10–12). The luminal membrane of MAL and CAL is impermeable to NH₃ (10,12). The nonionic diffusion of NH₃ into the lumen of OMCD, which is stimulated by low luminal pH, has been thought to be the primary route of ammonium excretion, at least under control conditions (18). NH₄⁺ has also been shown to substitute for K⁺ for NKCC1 (31). Wall (32) reported that the participation of NKCC1 in ammonium excretion in the OMCD is only 6% under control conditions. However, the upregulation of NKCC1 mRNA and protein by dehydration and metabolic

---

**Figure 6.** Rat NKCC1 protein expression in the kidney in control rats, rats with metabolic acidosis, and dehydrated rats. Membrane fractions from the cortex (CX), the outer medulla (OM), and the inner medulla (IM) were used for Western blotting. (Top) Typical example of a Western blot. (Bottom) Summary of NKCC1 protein expression in the three parts of the kidney, from nine experiments. NKCC1 protein abundance in the membrane fraction from the outer medulla was significantly increased by metabolic acidosis and dehydration. C, control; A, metabolic acidosis; D, dehydration. *P < 0.05 versus the control value for each part.

**Figure 7.** Rat NKCC1 protein expression in the OMCD and MAL in control rats, dehydrated rats, and rats with metabolic acidosis. Micro-dissected OMCD (20-mm long) and MAL (20-mm long) were used for Western blotting. (Top) Typical example of a Western blot. (Bottom) Summary of NKCC1 protein expression in OMCD and MAL, from seven experiments. NKCC1 protein bands at 170 kD were observed only in the OMCD and not in the MAL. NKCC1 protein expression in the OMCD was significantly stimulated by 2 d of dehydration and chronic metabolic acidosis. C, control; A, metabolic acidosis; D, dehydration. *P < 0.05 versus control.
acidosis suggests that the role of NKCC1 in dehydration and chronic metabolic acidosis should be much larger. When these findings are taken together, it can be speculated that NH₄⁺ is absorbed via NKCC2 in MAL and CAL and is secreted via nonionic diffusion and via NKCC1 in the OMCD. Whether the participation of NH₄⁺ secretion via NKCC1 in metabolic acidosis plays some role in ammonium secretion must be examined.

Our results showing the highest level of NKCC1 protein expression in the inner medulla of control rats differ slightly from the results of Ginnis et al. (17). Those authors reported the highest level of expression in the base region of the inner medulla and the inner stripe of the outer medulla. We combined the base and tip regions of the inner medulla and the outer and inner stripes of the outer medulla. This high level of expression in the inner medulla is thought to be caused by the difference in the populations of collecting ducts in the outer and inner medulla; the IMCD occupies approximately 20% of the inner medulla, whereas the OMCD occupies only 10% of the outer medulla (33). It is not known whether NKCC1 exists not only in IMCD but also in other types of cells, such as interstitial cells of the inner medulla. NKCC1 protein expression must be precisely examined by Western blot analysis using microdissected OMCD and IMCD.

It is interesting that dehydration stimulated both NKCC1 mRNA and protein expression in the OMCD in our study. Arterial blood gas analysis revealed that rats experience respiratory alkalosis under dehydration conditions. Plasma HCO₃⁻ concentrations were decreased in response to reduced P CO₂, and plasma vasopressin levels were significantly increased. Low plasma HCO₃⁻ concentrations, high blood vasopressin levels, or high osmolality in the medullary interstitium and urine in dehydrated rats could stimulate NKCC1 expression in the OMCD (34). Hypertonic cell shrinkage is known to stimulate rat parotid NKCC1 activity, suggesting a role for NKCC1 in acute regulatory volume increases (35). Chronic cell volume regulation takes place via the accumulation of osmolytes in the cells. The participation of NKCC1 in chronic cell volume regulation, however, is not known. Protein kinase inhibitors (specifically staurosporine and K252a) are known to blunt the stimulation, suggesting the participation of protein kinase C in NKCC1 activation (35). Volume-sensitive phosphorylation of NKCC1 by c-Jun amino-terminal kinase has also been reported (36). NKCC1 activity in the parotid gland is also increased by β-adrenergic and muscarinic stimulation (37,38). Recent reports unexpectedly revealed that mice lacking NKCC1 exhibit signs of deafness and imbalance (20,21). Although epithelial chloride secretion has been reported to be impaired, the characteristics of ion transport in the OMCD in such knockout animals are not yet known. The specific mechanisms of NKCC1 regulation in OMCD must be examined further.

In summary, NKCC1 mRNA and protein expression in OMCD is upregulated under conditions of dehydration and chronic metabolic acidosis. These data suggest that NKCC1 may play an important role in renal adaptation to these physiologic conditions. Low pH and possibly hypertonicity seem to stimulate NKCC1 mRNA expression.

Acknowledgments

We thank Dr. R. James Turner (National Institutes of Health, Bethesda, MD) for giving us the antibody against rat NKCC1. A part of this work was presented at the 30th Annual Meeting of the American Society of Nephrology (San Antonio, November 1997) and was published in abstract form (J Am Soc Nephrol 8: 35A, 1997). This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan (Grants 08671291, 09470238, 09557091, 10671000, 11470219, and 11877177) and by the Mochida Memorial Foundation for Medical and Pharmaceutical Research.

References


