Alkali Therapy Attenuates the Progression of Kidney Injury via Na/H Exchanger Inhibition in 5/6 Nephrectomized Rats

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Metabolic acidosis is a cause of renal disease progression, and alkali therapy ameliorates its progression. However, there are few reports on the role of renal acid-base transporters during alkali therapy. We evaluated the effect of sodium bicarbonate therapy and the role of acid-base transporters on renal disease progression in rats with a remnant kidney. Sprague-Dawley rats consumed dietary sodium bicarbonate ($NaHCO_3$) or sodium chloride (NaCl) with 20% casein after a 5/6 nephrectomy. After being provided with a casein diet, the $NaHCO_3$-treated group had higher levels of serum bicarbonate than the control group. At week 4, the glomerular filtration rate in the $NaHCO_3$ group was higher than that in the NaCl group, and the difference became prominent at week 10. The glomerulosclerosis and tubulointerstitial damage indices in the $NaHCO_3$ group were less severe compared with controls at week 4 and 10. The expression of the Na/H exchanger (NHE) was decreased, and apical reactivity was decreased in the $NaHCO_3$ group, compared with the NaCl group. Endothelin-1 levels in the kidney were also decreased in the $NaHCO_3$ group. Dietary sodium bicarbonate has the effects of ameliorating renal disease progression, which may be related to the altered expression of NHE in the remaining kidney.

Keywords: Sodium Bicarbonate; Acidosis; Kidney Failure, Chronic

INTRODUCTION

Metabolic acidosis is associated with the progression of chronic renal failure (CRF) and can be a common consequence of CRF (1). Conversely, many clinical studies have shown that metabolic acidosis itself may be a cause of renal disease progression and that alkali therapy ameliorates its progression (2, 3). In experimental studies, several mechanisms for the effects of alkali therapy have been evaluated, such as the reduced activation of the alternative complement pathway (4), decreased cell proliferation and transdifferentiation (5), and the endothelin (ET) antagonism pathway (6). However, there are few reports of the role of renal acid-base transporters during alkali therapy in remnant kidney models.

Luminal Na/H exchanger type 3 (NHE3) is responsible for a significant portion of renal Na^+ and HCO_3^- absorption (7). NHE3 is variably expressed depending on the pathologic condition and was increased in adriamycin-nephropathy models (8). NHE3 activation plays a role in the pathogenesis of isch-

emic acute renal failure (9). Under alkali loading, apical NHE3 expression in the kidney can be down-regulated (10). The inhibition of NHE3 may improve kidney function and structure in acute ischemic kidney injuries (11). However, there are few reports of the effects of alkali therapy on apical NHE3 expression in chronic kidney failure. We evaluated the beneficial effects of dietary sodium bicarbonate in ameliorating renal disease progression and the role of NHE3 with regard to these effects in rats with a remnant kidney.

MATERIALS AND METHODS

Experimental animals

Twenty-three specific pathogen-free male Sprague-Dawley rats (5-6 weeks old, 160-190 g; Orient Bio Inc., Seongnam, Korea) were housed with free access to water. Experimental CRF was induced by the excision of approximately two-thirds of the left kidney and a right total nephrectomy. After a 5/6 nephrectomy, all rats were randomly allocated to the NaCl-treated group or

the NaHCO₃-treated group. CRF rats in the NaHCO₃-treated group were given a 20% casein diet with 174 μM/g NaHCO₃ (DYET #113316, Dyets Inc., Bethlehem, PA, USA) and were considered alkali-treated animals. CRF rats in the NaCl-treated group were given a 20% casein diet with 174 μM/g NaCl (DYET #113811, Dyets Inc.) and were considered control animals treated with the same amount of dietary sodium. The NaHCO₃ and NaCl amounts were determined according to the previous report (6).

CRF rats in the two groups were euthanized at the time points of 4 and 10 weeks after the operations (n = 10 at 4 weeks and n = 13 at 10 weeks). Fragments of remnant kidneys were taken from center areas and divided into two parts. One part was used for protein extraction, and the other for pathology examination. The glomerular sclerosis (GS) index was assessed in 50 glomeruli per rat on periodic-acid Schiff-stained paraffin sections at a magnification of 400 × (12). The tubulointerstitial (TI) lesion indices were determined using a semiquantitative scoring system on Masson's trichrome-stained sections at a magnification of 100 × (5). The pathologic scores for GS and TI were performed in blinded fashion.

Physiologic data

Blood pressure was measured using the tail-cuff plethysmography method (IITC Life Sciences, Woodland Hills, CA, USA). For the final 2 days prior to euthanasia, the animals were placed in metabolic cages, and 24-hr urine samples were collected on the day before euthanasia for the measurement of urine urea nitrogen (UUN), creatinine (Cr), protein, osmolality, Na, K, and ET-1 levels. A blood sample was collected from the abdominal aorta at the time of euthanasia for the measurement of blood urea nitrogen (BUN), Cr, osmolality, and electrolyte levels. Biochemical tests were performed using an automatic chemistry analyzer (Hitachi 7070; Hitachi, Tokyo, Japan). Indirect ion-selective electrode methods were used to obtain electrolyte levels, and the enzyme method was used for measuring total CO₂ levels. Plasma and urine osmolality were measured with a cryoscopic osmometer (Osmomat 030-D-M; Gonotec, Berlin, Germany). The glomerular filtration rate (GFR) was calculated using the average of urea clearance and creatinine clearance (7).

Semiquantitative immunoblotting

The remnant kidneys were quickly removed and placed in a chilled isolation solution containing 250 mM sucrose, 10 mM triethanolamine (Sigma, St. Louis, MO, USA), 1 ng/mL leupeptin (Sigma), and 0.1 mg/mL phenylmethylsulfonyl fluoride (Sigma), titrated to pH 7.6. The pieces were homogenized at 15,000 rpm with three strokes for 15 sec using a tissue homogenizer (Power-Gun 125; Fisher Scientific, Pittsburgh, PA, USA). After homogenization, the total protein concentration of the homogenate was measured by the bicinchoninic acid protein assay method (BCA

Reagent Kit; Sigma) and diluted to 2.05 μg/μL using the isolation buffer solution. The samples were then stabilized by heating to 60°C for 15 min after adding 1 vol 5 × Laemmli sample buffer/4 vol sample. Initially, loading gels were used with each sample set. Five micrograms of protein from each sample were loaded into individual lanes, run on 12% polyacrylamide-SDS minigels using a Mini Protean III electrophoresis apparatus (Bio-Rad, Hercules, CA, USA) and stained with Coomassie blue dye (G-250, Bio-Rad; 0.025% solution prepared in 4.5% methanol and 1% acetic acid), which were considered internal standards (13). 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 into each lane. Finally, protein concentrations were corrected to reflect these measurements and were subjected to immunoblot analysis, using a previously described method (7).

Immunohistochemistry

The other half of the left kidney from each rat was immersed in a 4% paraformaldehyde solution at 4°C overnight. Each slice was dehydrated with a graded series of ethanol and embedded in paraffin. The embedded pieces of kidney were sectioned at a 3 μm thickness on a microtome (RM 2145; Leica Instruments GmbH, Nussloch, Germany) and mounted on gelatin-coated glass slides. The sections were deparaffinized with xylene, dehydrated with a graded series of ethanol, and rehydrated. Antigen retrieval was performed by repeated boiling and cooling in a citric acid buffer. Endogenous peroxidase activity was blocked by incubation of the sections in 3% H₂O₂ for 10 min. Next, the immunostaining procedure was performed according to the protocol of the Dako Cytomation kit (Envision + Dual Link System-HRP; Carpinteria, CA, USA). The sections were incubated for 1 hr with antibody against NHE3 and Na-K-ATPase at room temperature.

Primary antibodies and cytokines assay

For semiquantitative immunoblotting and immunohistochemistry, we used previously characterized polyclonal antibodies. Affinity-purified polyclonal antibodies against NHE3, NKCC2, NCC, ENaC-α, ENaC-γ, NBC, pendrin, and H-ATPase were used as described in a previous study (7, 14). Affinity-purified polyclonal antibodies against Na-glucose cotransporter-1 (SGLT1; AB1352; Millipore Corp., Billerica, MA, USA), ENaC-ß (sc-21013; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and Na-K-ATPase (05-396; Millipore Corp.) were also used. Tissue ET-1 concentrations were measured using QuantiGlo ET-1 ELISA (QET00B; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Statistical analysis

Comparisons between the two groups were made with the Mann-

Whitney U-test, and comparisons among three groups were made with the Kruskal-Wallis test (SPSS software; SPSS Inc., Chicago, IL, USA). Band density values were standardized by dividing them by the average value of the control group. The mean for the control group was defined as 100%, and the results were expressed as the means ± SEM. $P < 0.05$ was considered statistically significant.

Ethics statement

The institutional animal care and use committee at Seoul National University Hospital approved the experimental protocol (No.10-0192).

RESULTS

Change of Physiologic and Pathologic Data

After a 5/6 nephrectomy, serum total CO_2 levels in the $NaHCO_3$-treated group were higher compared with the control group throughout the study period (Table 1). At 4 weeks, the GFR in the $NaHCO_3$ group was slightly higher than that in the NaCl group, although it was not statistically significant. At week 10, the GFR in the $NaHCO_3$-treated group was significantly higher than that in the control group. GFRs between week 4 and week 10 remained constant in the $NaHCO_3$-treated group, while GFRs decreased in the control group. The degree of proteinuria and the systolic blood pressure were similar at week 4 and week 10 between the groups consuming NaCl and $NaHCO_3$. Fig. 1 shows

the dietary Na intake per day (input), urinary Na excretion per day (output), and Na balance (the difference between input and output). There were no differences in the Na balance between the two groups at week 4 and week 10. At week 4, the GS index in the $NaHCO_3$-treated group was less severe compared to the NaCl-treated group (0.17 ± 0.041 vs 0.47 ± 0.063, respectively, $P < 0.001$) (Fig. 2). These differences persisted at week 10 (0.66 ± 0.063 in the $NaHCO_3$-treated group vs 0.99 ± 0.074 in the NaCl-treated group, $P = 0.001$). At week 4, the TI damage index in the $NaHCO_3$-treated group was half of that in the NaCl-treated group (0.45 ± 0.010 vs 0.92 ± 0.120, respectively, $P = 0.004$). Similarly, the TI damage index in the $NaHCO_3$-treated group was 3/4-fold of that in the NaCl-treated group (1.33 ± 0.12 vs 1.80 ± 0.12, respectively, $P = 0.010$). Both GS and TI damage indices gradually progressed between week 4 and week 10 in the two groups.

Change of renal transporters

Fig. 3 shows the immunoblots for renal Na and acid-base transporters. The expression of NHE3 in the $NaHCO_3$-treated group was significantly decreased compared to the control group at week 4 (10.1 ± 4.25 vs 100 ± 21.1, respectively, $P = 0.007$). At week 10, the expression of NHE3 in the $NaHCO_3$-treated group was decreased, compared to the control group; however, the decrease was not statistically significant (37.1 ± 13.0 vs 100 ± 52.9, $P = 0.308$). Immunohistochemistry revealed that apical expression for NHE3 was more prominent in the NaCl-treated group

Table 1. Physiologic data in $NaHCO_3$-treated and NaCl-treated chronic renal failure (CRF) rats each period

Parameters	Week 0		Week 4		Week 10	
	CRF + NaHCO₃ (n = 10)	CRF + NaCl (n = 13)	CRF + NaHCO₃ (n = 4)	CRF + NaCl (n = 6)	CRF + NaHCO₃ (n = 6)	CRF + NaCl (n = 7)
CCr (mL/min/100 g BW)	0.61 ± 0.04	0.61 ± 0.05	0.21 ± 0.03	0.17 ± 0.01	0.24 ± 0.01	0.16 ± 0.02*
Curea (mL/min/100 g BW)	0.51 ± 0.05	0.55 ± 0.05	0.10 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.06 ± 0.01
GFR ([CCr+Curea]/2)	0.56 ± 0.04	0.58 ± 0.04	0.16 ± 0.02	0.13 ± 0.01	0.16 ± 0.01	0.11 ± 0.01*
Blood urea nitrogen (mg/dL)	15.5 ± 1.38	12.6 ± 0.91	71.4 ± 14.3	68.0 ± 5.25	52.0 ± 7.71	103 ± 21.0
Creatinine (mg/dL)	0.50 ± 0.01	0.50 ± 0.03	1.230 ± 0.21	1.20 ± 0.01	1.01 ± 0.06	1.62 ± 0.27*
BW (g)	188 ± 1.96	184 ± 2.67	378 ± 11.7	374 ± 4.76	439 ± 11.6	413 ± 17.6
Systolic blood pressure (mmHg)	108 ± 2.01	111 ± 1.51	166 ± 2.41	165 ± 2.62	193 ± 2.65	195 ± 3.75
Urine Protein/creatinine (g/g)	0.41 ± 0.11	0.50 ± 0.09	19.8 ± 1.62	24.1 ± 7.36	16.3 ± 1.73	16.7 ± 1.41
Serum Na (mM/L)	149 ± 1.01	151 ± 0.75	145 ± 0.64	145 ± 1.58	143 ± 0.31	144 ± 1.00
Serum K (mM/L)	3.89 ± 0.11	3.61 ± 0.13	5.11 ± 0.33	4.74 ± 0.10	5.38 ± 0.08	6.27 ± 0.45*
Serum tCO₂ (mM/L)	21.2 ± 1.07	21.7 ± 0.77	28.6 ± 0.65	20.9 ± 1.29†	23.5 ± 0.53	16.1 ± 0.89*
FENa (%)	1.07 ± 0.07	1.05 ± 0.09	3.97 ± 0.84	3.15 ± 0.22	1.94 ± 0.30	2.45 ± 0.22*
FEK (%)	57.2 ± 3.98	58.2 ± 3.64	40.5 ± 6.51	39.0 ± 2.48	28.3 ± 5.38	28.0 ± 2.62
Urine flow (mL/hr)	0.47 ± 0.02	0.50 ± 0.04	1.81 ± 0.20	1.76 ± 0.12	1.49 ± 0.21	2.48 ± 0.15*
Urine osm (Osm/kg)	1484 ± 76.7	1310 ± 98.0	517 ± 64.2	540 ± 43.3	607 ± 113	473 ± 57.0*
UNa X UV (μM/min/100 g BW)	0.97 ± 0.03	0.94 ± 0.06	0.84 ± 0.05	0.81 ± 0.03	0.50 ± 0.02	0.73 ± 0.08

Values are presented as the means ± SEM. *$P < 0.05$; †$P < 0.01$. CCr, creatinine clearance; Curea, urea clearance; BW, body weight; FENa, fractional excretion of sodium; FEK, fractional excretion of potassium; UV, urine volume.

than in the NaHCO$_3$-treated group at week 4 and week 10.

Na-K-ATPase expression in the NaHCO$_3$-treated group was
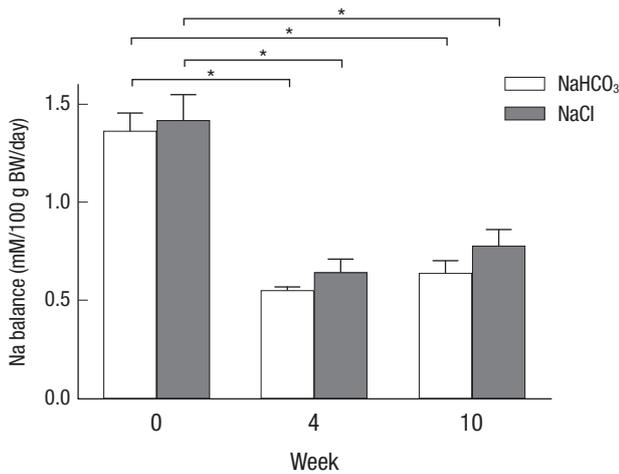
Fig. 1. Daily Na balance in NaHCO$_3$-treated and NaCl-treated rats for each period. There are no differences between the two groups for each period. Data are presented as the means ± SEM.

significantly decreased at week 4 compared to the NaCl-treated group (57.8 ± 13.0 vs 100 ± 11.5, respectively, P = 0.025). There was no difference in the expression of Na-K-ATPase between the two groups at week 10. On immunohistochemistry, the basolateral expression for Na-K-ATPase was increased in the NaCl-treated group at both week 4 and week 10 (Fig. 4).

There was no change in the densities of NKCC2, SGLT1, H-ATPase, NBC, and pendrin in the two groups at week 4 and week 10. The densities of NCC increased and those of ENaC-α decreased in the NaHCO$_3$-treated animals at week 10.

At week 4, there was no difference in the renal ET-1 levels between the NaHCO$_3$-treated group and the NaCl-treated group (0.18 ± 0.096 vs 0.15 ± 0.019 [pg/mL]/[mg/mL], respectively) (Fig. 5). In contrast, the renal ET-1 levels in the NaHCO$_3$-treated group were decreased at week 10, compared with the NaCl-treated group (0.32 ± 0.15 vs 1.14 ± 0.20 [pg/mL]/[mg/mL], respectively, P = 0.021). In addition, the NaCl-treated rats were increased at week 10, compared with the same group at week 4 (P = 0.011).
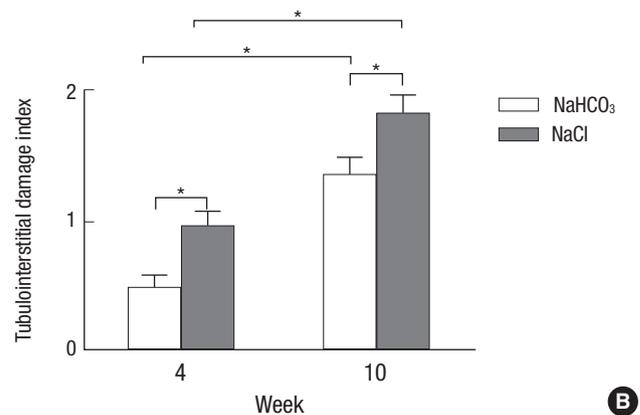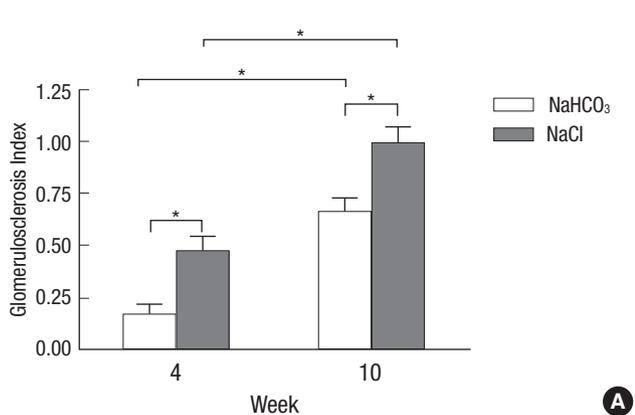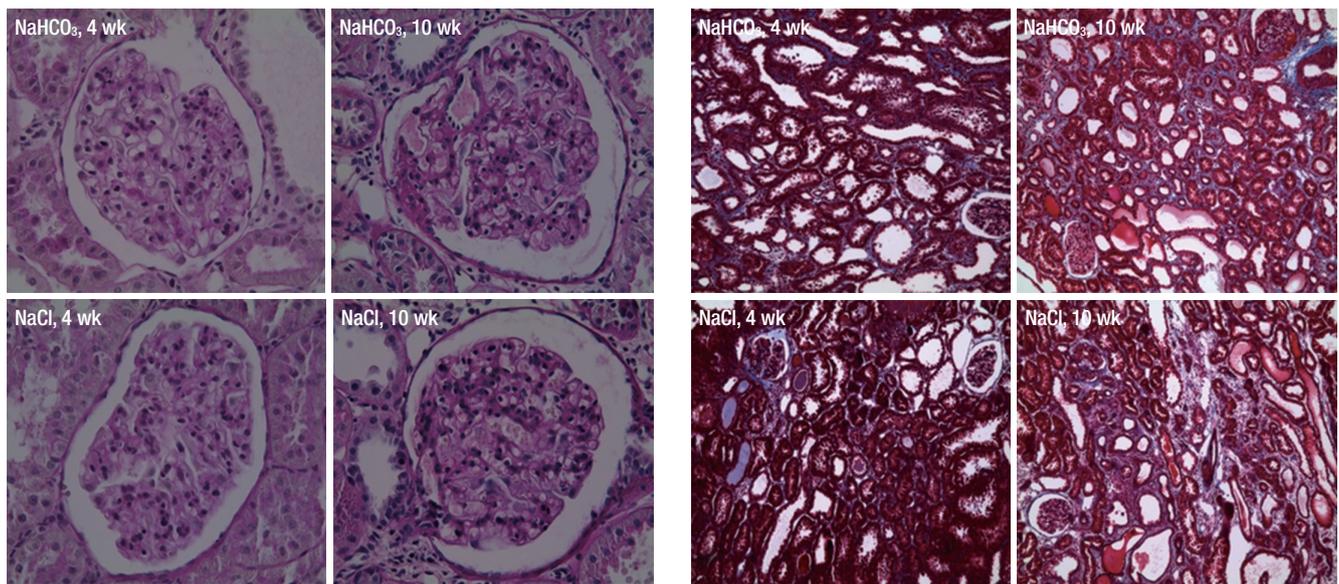

Fig. 2. Histological analysis of remnant kidney tissue. Histological sections include glomerular lesions (**A**) on periodic-acid Schiff-stained sections at a magnification of × 400 and tubulointerstitial lesions (**B**) on Masson's trichrome stained sections at a magnification of × 100. Data are presented as the means ± SEM. *P < 0.05.
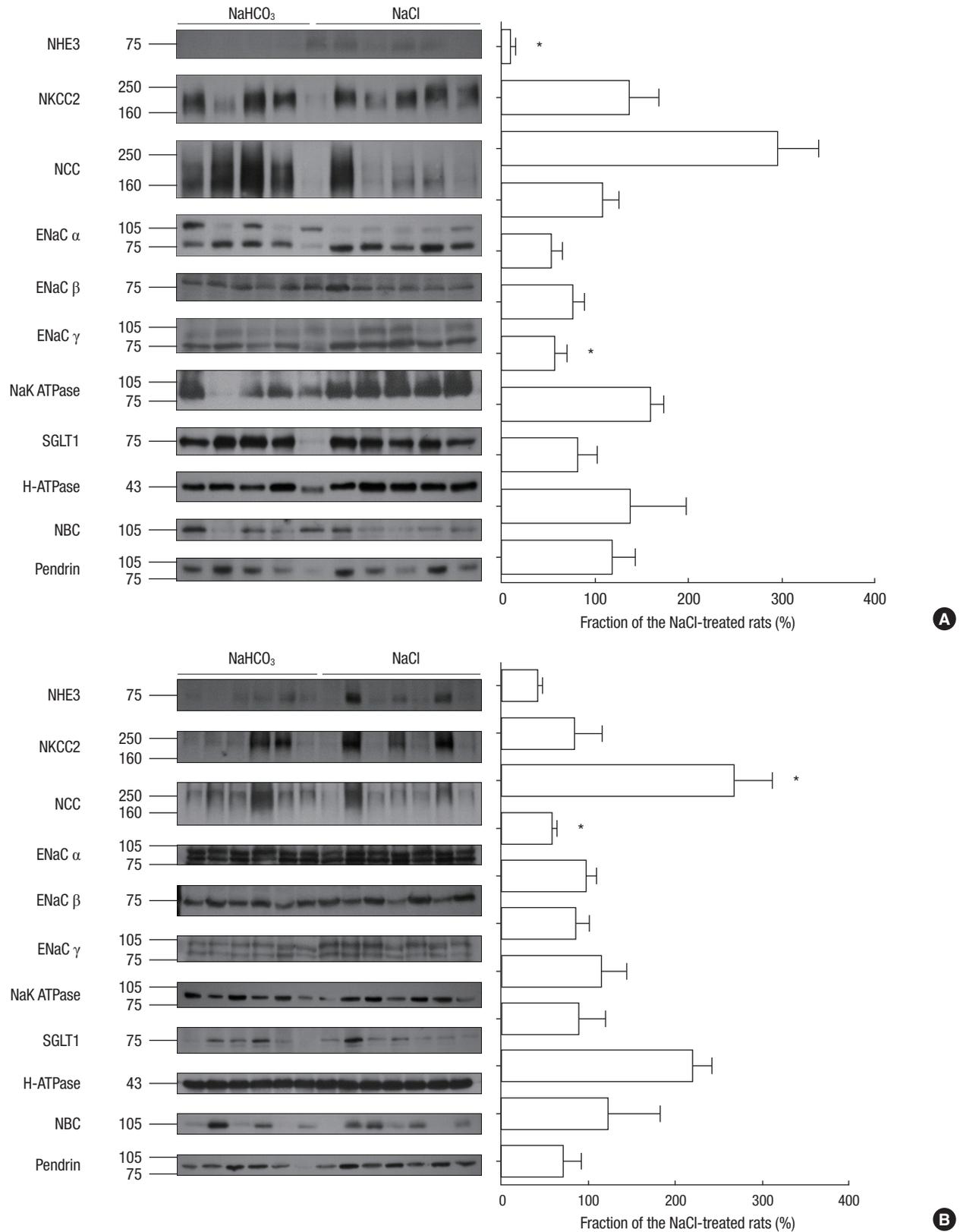
Fig. 3. Immunoblot (left) and densitometric (right) analysis of renal Na transporters and acid-base transporters from NaHCO₃- and NaCl-treated rats at week 4 (**A**) and week 10 (**B**) after nephrectomy. The dotted line indicates the levels of NaCl-treated animals as controls. Data are presented as the means ± SEM. *$P < 0.05$ for NaHCO₃-treated rats compared with the NaCl-treated rats for the same period.
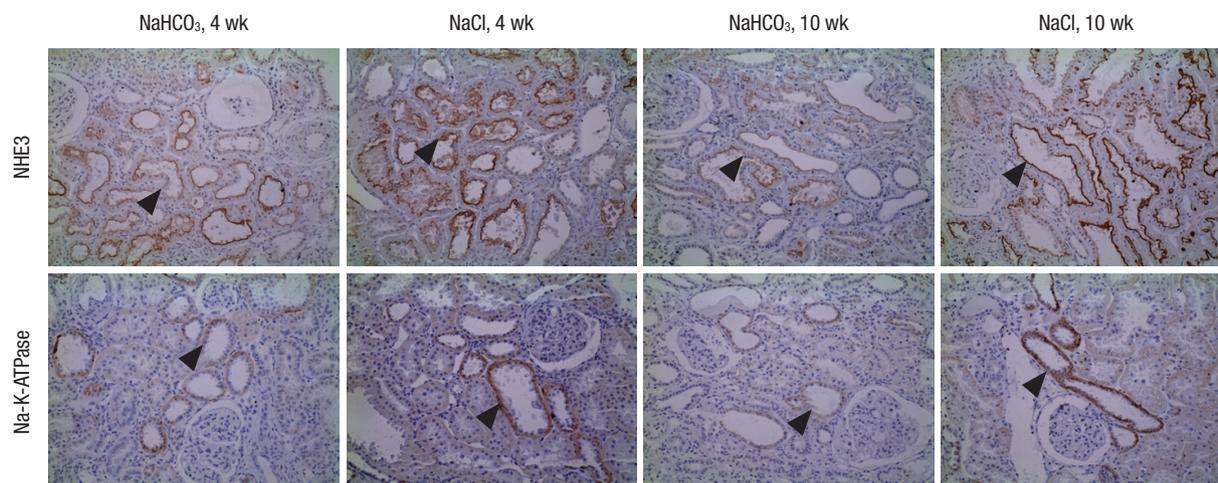
Fig. 4. Immunohistochemical analysis of NHE3 and Na-K-ATPase. The immunostaining of apical membrane of NHE3 in NaHCO₃-treated rats is decreased, compared to that in NaCl-treated rats at week 4 and week 10. Additionally, the labeling density of the basolateral border of Na-K-ATPase in NaHCO₃-treated rats is reduced compared with that in control rats at week 4 and week 10. × 200.
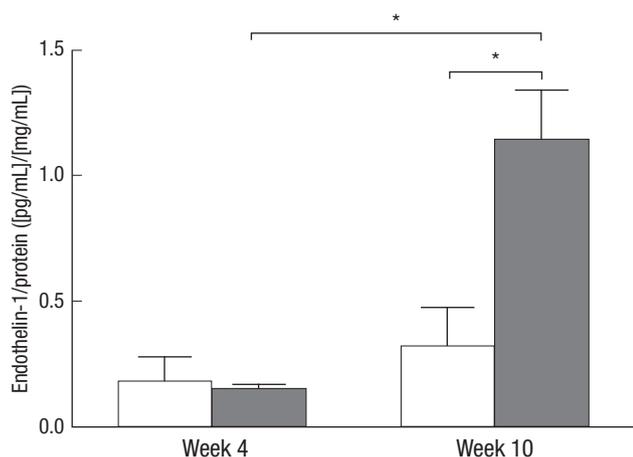
Fig. 5. Kidney tissue levels of endothelin-1 in NaHCO₃-treated (white box) and NaCl-treated (gray box) rats for each period. Data are presented as the means ± SEM. *P < 0.05.

DISCUSSION

Our data show that dietary sodium bicarbonate in the nephrectomized models may have beneficial effects in ameliorating the decrease in GFR and pathologic damage. Our data also show that these effects may be associated with NHE3 expression as well as ET-1 levels. NHE activity in the myocardium is associated with cardiac remodeling (15), and NHE inhibition may lead to the regression of myocardial fibrosis (16). Renal NHE expression was upregulated in adriamycin-induced nephropathy in parallel with the degree of glomerulosclerosis and interstitial fibrosis, and the preventive effects of amiloride on renal lesions suggest the potential importance of NHE (17). The inhibition of NHE may be beneficial for protection in cases of decreased kidney function as well as tubular injury in acute kidney injury (11). This study provides evidence that NHE3 inhibition may be associated with renal protective effects in CRF.

Chronic metabolic acidosis induced by acid loading enhances NHE3 protein abundance and transport activity in the rat thick ascending limb (18). After the correction of metabolic acidosis with sodium bicarbonate in our experiment, NHE3 expression was decreased compared with the control group. NaHCO₃ loading can directly downregulate apical NHE3 expression in the rat kidney proximal tubule (10). The downregulation of NHE3 could be responsible for a decreased acid burden due to the correction of metabolic acidosis and increased excretion of alkaline excess in nephrectomized rats subjected to NaHCO₃ loading.

In the previous study, we evaluated the expression of renal tubular transporters in 5/6 nephrectomized rats with a regular diet (7). Increased urinary sodium excretion was associated with decreased expression of renal sodium transporters, especially NHE3 in the proximal tubule. There was no difference between the two groups in terms of sodium loading and sodium balance at week 4 and week 10, but NHE expression in the NaHCO₃-treated group was decreased more than in the NaCl-treated group. This suggests that the downregulation of NHE3 may be affected by alkali loading independent of sodium loading in CRF. In contrast, the expression of H-ATPase, NBC, or pendrin, which are major regulators of acid-base homeostasis, may not be associated with alkali therapy in CRF rats. Therefore, NHE3 may be a main target of bicarbonate therapy.

Augmented intrinsic acid production promotes TI injury through endothelin receptors (19). Chronic metabolic acidosis induces increased ET expression in the renal proximal tubule (20, 21). Moreover, ET expressed by the kidney can activate proximal tubule acidification by activating the proximal tubule NHE, while ET has a lack of effects on the activities of the apical SGLT (22). This effect of ET has been shown to involve the traf-

ficking of NHE3 to the apical membrane, which is achieved by an increase in the exocytic insertion of NHE3 into the apical membrane (21, 23). In our study, apical membrane NHE abundance was decreased in the alkali-treated group with relatively decreased ET levels.

Chronic metabolic acidosis may stimulate NKCC2 of the rat medullary thick ascending limb (24). In our data, NKCC2 expression was not different between the NaHCO₃- and NaCl-treated groups. This may be related to the decreased expression of NHE3, which may be associated with increased distal sodium delivery. NH₄Cl-induced metabolic acidosis in rats is associated with a strong downregulation of NCC in the distal convoluted tubules (25). In our data, NCC expression tended to be increased in the NaHCO₃-treated group, compared with the NaCl-treated group. It is possible that the expression of NCC, decreased in acidic condition, may be restored under the alkali therapy.

When NHE3 was stimulated by feeding NH₄Cl, Na-K-ATPase activity was increased (26). When NHE3 was inhibited by the administration of amiloride, however, Na-K-ATPase activity was decreased in rat kidney proximal convoluted tubule segments (26). In our data, Na-K-ATPase expression was decreased at week 4, and the basolateral reactivity of the Na-K-ATPase was also decreased at week 4 and week 10 in the NaHCO₃-treated group. The change of Na-K-ATPase activity may be affected by altered NHE3 expression.

Acidification inhibits ENaC activity, whereas alkalinization stimulates ENaC (27). In our data, most ENaC abundance was not changed in both groups, although α-ENaC density was decreased in the NaHCO₃-treated rats at week 10. In rats with normal renal function, NaHCO₃ or NaCl loading downregulates basolateral NBC (10). In our nephrectomized rats, NaHCO₃ loading did not affect NBC expression. In addition, the expression of pendrin or H-ATPase in the distal tubule did not change through the correction of acidosis. It is possible that renal acid-base transporters under CRF conditions could be regulated by a different mechanism. The similar expression of pendrin in two CRF groups may lead to ENaC, because pendrin modulates ENaC abundance under luminal alkalinization (28).

Phisitkul et al. (6) demonstrated the changes of GFR in 5/6 nephrectomized rats with a variety of diet and alkali therapy. The NaHCO₃ treatment that was under the control of blood pressure showed better GFR outcomes than NaHCO₃ treatment alone. In our study, we did not give any anti-hypertensive medication to the two groups. In both groups, high blood pressures may promote GS and/or TI damage and altered expression of renal tubular transporters, although there was no difference in the blood pressure in the two groups. Further experiments on the role of renal transporters in CRF rats under the control of blood pressure should be performed.

In summary, dietary sodium bicarbonate intake contributed

to decrease of NHE3 and ET levels. Expression of Na-K-ATPase and NCC in CRF rats may be directly or indirectly related to the alteration of NHE abundance. This finding suggests that alkali treatment in CRF may have beneficial effects in ameliorating renal disease progression, and these effects may be mainly associated with the altered expression of NHE3 in the kidney, which may be a therapeutic target for renal disease progression.

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DISCLOSURE

The authors have declared no conflicts of interest.

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