Effect of high-NaCl or high-KCl diet on hepatic Na\(^+\)- and K\(^+\)-receptor sensitivity and NKCC1 expression in rats

You Tsuchiya, 1,2 Shigeru Nakashima, 3 Yoshiko Banno, Yuichi Suzuki, 2 and Hironobu Morita 1

1Department of Physiology and 2Department of Biochemistry, Gifu University School of Medicine, Gifu 500–8705; and 3Department of Food and Nutritional Sciences, Shizuoka Prefectural University, Shizuoka 422–8526, Japan

Submitted 26 September 2003; accepted in final form 27 November 2003

Tsukiyama, You, Shigeru Nakashima, Yoshiko Banno, Yuichi Suzuki, and Hironobu Morita. Effect of high-NaCl or high-KCl diet on hepatic Na\(^+\)- and K\(^+\)-receptor sensitivity and NKCC1 expression in rats. *Am J Physiol Regul Integr Comp Physiol* 286: R591–R596, 2004. First published December 4, 2003; 10.1152/ajpregu.00559.2003.—We previously reported that the bumetanide-sensitive Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter (NKCC1) is involved in the hepatic Na\(^+\) and K\(^+\) sensor mechanism. In the present study, we examined the effects of a high-NaCl or high-KCl diet on hepatic Na\(^+\) and K\(^+\) receptor sensitivity and NKCC1 expression in the liver of Sprague-Dawley rats. RT-PCR and Western blots were used to measure NKCC1 mRNA and protein expression, respectively. Infusion of hypertonic NaCl or isotonic KCl + NaCl solutions into the portal vein increased hepatic afferent nerve activity (HANA) in a Na\(^+\) or K\(^+\) dose-dependent manner. After 4 wk on a high-NaCl or high-KCl diet, HANA responses were attenuated compared with animals fed a normal diet, and NKCC1 expression was reduced. These results show that a high-NaCl or high-KCl diet decreases NKCC1 expression in the liver, and it might cause a reduction in hepatic Na\(^+\) and K\(^+\) receptor sensitivity.

CONTINUOUSLY EXPOSED TO high concentrations of Na\(^+\) and K\(^+\), this may modulate the expression and sensitivity of the receptors. To test this hypothesis, NKCC1 mRNA and protein expression and the sensitivity of hepatic Na\(^+\) and K\(^+\) receptors were measured after 4 wk on a high-NaCl or high-KCl diet.

MATERIALS AND METHODS

The experiments were performed on male Sprague-Dawley rats weighing 300–360 g (n = 48). The animals were maintained in accordance with the “Guiding Principles for Care and Use of Animals in the Field of Physiological Science” of the Physiological Society of Japan at constant humidity (60 ± 5%) and temperature (23 ± 1°C) and on a light cycle (lights on 0700–1900). The rats (7 wk old) were assigned randomly to three experimental groups and were put on a normal diet (0.3% Na\(^+\), 0.8% K\(^+\)), high-NaCl diet (3% Na\(^+\), 0.8% K\(^+\)), or high-KCl diet (0.3% Na\(^+\), 8% K\(^+\)) for 4 wk. All experiments were performed during the light period. Before experiments, rats were fasted overnight with free access to water. At the end of the diet period, HANA measurements were made on eight rats from each group, while another eight were anesthetized with pentobarbital sodium (50 mg/kg ip) and intracardially perfused with heparinized saline; the liver was rapidly removed and cut into pieces, which were immersed in liquid nitrogen and stored at −30°C until use for RT-PCR and Western blots.

Measurement of HANA (8 rats from each group). The rats were anesthetized with pentobarbital sodium (50 mg/kg ip), and a venous catheter was inserted into the inferior vena cava via the femoral vein for blood sample and infusion of any required supplementary doses of anesthetic. Using central laparotomy, a portal venous catheter was inserted into the mesenteric vein. The perirrenal hepatic nerve was isolated and two stainless steel electrodes (model 7901; A-M Systems, Everett, WA) were placed around it; the nerve and electrodes were then covered and held together with silicone gel (604A and 604B, Wacker Chemie, Munich, Germany). The proximal side of the hepatic nerve was then ligated, and afferent nerve activity was recorded.

The electrical activity recorded from the hepatic nerve was amplified using a 50-Hz to 1-kHz band-pass filter (model AVB-10, Nihon Kohden, Tokyo), the amplifier output was rectified by an absolute value circuit, and the rectified signal was integrated arithmetically. The original waveform and the rectified signal were sampled using an analog-to-digital converter (mode MP100, Biopac Systems, Goleta, CA) at a rate of 100 samples/s. At the end of preparation, 1 ml of venous blood sample for measuring plasma Na\(^+\), K\(^+\), and Cl\(^-\) was obtained.

While measuring HANA, hypertonic NaCl solutions (0.375, 0.75, and 1.5 M) and various isotonic KCl + NaCl solutions (25 mM KCl + 125 mM NaCl, 50 mM KCl + 100 mM NaCl, and 100 mM KCl + 50 mM NaCl) were injected as bolus doses of 0.1, 0.2, 0.5, and 1.0 ml/kg body wt via the portal venous catheter. Each animal was injected with all concentrations of all solutions in a randomized order. No further injections were performed until the effect of the previous one dissipated.
injection on HANA was no longer apparent (~1 min). To quantify HANA responses, the rectified signal averaged over the 5 s immediately before each injection was taken as the 100% level. The peak value induced by injection was averaged over the 2 s centered on the peak and the response was presented as a percentage of the basal rate.

RT-PCR (8 rats from each group). Total RNA was isolated from the liver using the guanidinium thiocyanate-phenol-chloroform method (4). Briefly, the tissue was homogenized in 1 ml of ISOGEN (Nippon Gene, Tokyo), and the homogenate was centrifuged at 4°C for 10 min at 10,000 g. The supernatant was removed and mixed with 0.2 ml of chloroform, and then the sample was centrifuged at 4°C for 15 min at 12,000 g. Isopropanol (0.5 ml) was then added to the supernatant, and the sample was centrifuged at 4°C for 15 min at 12,000 g. The precipitate was suspended in 1 ml of cold 70% ethanol and centrifuged at 4°C for 5 min at 7,500 g; then the final precipitate was suspended in 50 μl of RNase-free distilled deionized water and used as the total RNA. Total RNA (1 μl) was mixed in a final volume of 25 μl with 1.5 μl of random hexamers and diethylpyrocarbonate-treated distilled water and incubated for 10 min at 65 °C. After incubation on ice for 5 min, the sample was reverse-transcribed for 60 min at 42°C in a total volume of 40 μl containing 8 μl × 5 RT buffer, 1 μl of reverse transcriptase (M-MLV RT RNaseH Minus, 200 U/μl, Promega Biological, Shanghai, China), and 8 μl of 2.5 mM dNTP, and then was amplified using the primers 5’-ATAGGAATGCAGTGTCGTTTAGG and 5’-TGGAGCAATGACATTCAATTCG, corresponding, respectively, to the 7th and 9th-10th transmembrane-spanning domains of rat parotid NKCC1 (15). The cyclophilin primer was used as a control for the RT-PCR reaction. The PCR reaction mixture contained 14 μl of water, 1 μl of cDNA (10 ng RNA), 1 μl of 100 μM primers, 2 μl of reaction buffer (×10), 2 μl of 2.5 mM dNTP, and 0.1 μl of 5 U/μl Taq DNA polymerase. The cycling conditions were 94°C for 30 s, 64°C for 1 min (58°C for cyclophilin), and 72°C for 1 min, using 26 cycles (30 cycles for cyclophilin). The reaction mixture was separated on a 1% agarose gel, and the bands were visualized by ethidium bromide staining. Semiquantification was performed by measuring the density of the bands on a densitometer.

Western blots (8 rats from each group; same animals as for the RT-PCR). The liver was homogenized in 0.5 ml of ice-cold buffer containing 25 μl of HEPES (1 M, pH 7.4), 6.25 μl of EGTA (400 mM), 1 μl of MgCl2 (1 M), 20.1 mg of mannitol, 11.6 mg of sucrose, and 0.5 μl of protease inhibitor E64 (Sigma, Tokyo). The homogenate was centrifuged at 800 g for 10 min at 4°C, and the supernatant was centrifuged at 1 h at 224,000 g for 4°C. The pellet was dissolved in extraction solution (1% Triton X-100, 0.1% SDS, 10 mM EDTA, 50 mM Tris-HCl, 1% Na-DOC, 150 mM NaCl, pH 8.8), and the protein content was estimated using a DC Protein Assay Kit (Bio-Rad). A sample containing 100 μg of protein was mixed with SDS sample buffer, heated at 95°C for 3 min, and electrophoresed overnight on a

Table 1. Body weight, hematocrit, and plasma Na⁺, K⁺, and Cl⁻ concentration after 4 wk of dietary period

<table>
<thead>
<tr>
<th></th>
<th>Normal Diet</th>
<th>High-NaCl Diet</th>
<th>High-KCl Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight, g (n = 16)</strong></td>
<td>343±4</td>
<td>334±5</td>
<td>328±5</td>
</tr>
<tr>
<td><strong>Hematocrit, % (n = 8)</strong></td>
<td>44±1</td>
<td>43±1</td>
<td>42±1</td>
</tr>
<tr>
<td><strong>Na⁺, meq/l (n = 8)</strong></td>
<td>145.0±0.7</td>
<td>144.8±0.7</td>
<td>145.6±0.6</td>
</tr>
<tr>
<td><strong>K⁺, meq/l (n = 8)</strong></td>
<td>3.3±0.1</td>
<td>3.3±0.1</td>
<td>3.2±0.1</td>
</tr>
<tr>
<td><strong>Cl⁻, meq/l (n = 8)</strong></td>
<td>104.6±0.5</td>
<td>103.3±0.8</td>
<td>103.7±0.6</td>
</tr>
</tbody>
</table>

Values are means ± SE.
7.5% SDS-PAGE gel, and then the proteins were electrophoretically transferred to nitrocellulose membranes. The membrane was blocked for 4 h at room temperature in blocking buffer consisting of Tris-buffered saline-Tween (TBST), 8% 1 M Tris-HCl, pH 7.4, 0.274 mM NaCl, 2 g/l Tween 20, and 123 mM azide, containing 5% bovine serum albumin, and then incubated 2 h at room temperature with blocking buffer containing a 1/1,500 dilution of T4 monoclonal antibody raised against the 310 COOH-terminal residues of human colonic NKCC1 (Zymed Laboratories, CA) (14). The membrane was then washed with TBST buffer and incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-mouse IgG. After extensive washing with TBST buffer, bound antibody was visualized by chemiluminescence (Western blotting detection reagent kit, Amersham Pharmacia Biotech, LCB).

Statistical analysis. All values are presented as means ± SE. The data were analyzed by one-way ANOVA, with diet as a factor. If the F ratio indicated statistical significance, Scheffe’s post hoc test was applied to compare between-group means. The significance level of the post hoc comparisons was set at P < 0.05.

RESULTS

HANA. After 4 wk of dietary period, no difference was observed in plasma Na⁺, K⁺, and Cl⁻ concentration among groups (Table 1). Figure 1A shows typical recording of HANA in response to bolus intraportal injection of 0.75 M NaCl (0.5 ml/kg body wt) in rats on a normal, high-NaCl, or high-KCl diet. In the rats on a normal diet, HANA increased with a short latency, elevated for a few seconds, and then returned to the baseline level. This response was reduced in rats on either a high-NaCl or high-KCl diet. The summarized data for the peak responses to different volumes of three different hypertonic NaCl solutions are presented in Fig. 1, B–D. In the normal diet group, intraportal injection of hypertonic NaCl solution caused the HANA to increase in a dose-dependent manner, i.e., increasing injected volume or concentration increased HANA response. However, the effect of an increased volume would not depend on volume itself because the volume-dependent response of HANA was not seen in case of intraportal injection of isotonic NaCl or hypertonic LiCl (17, 28). Thus the volume-dependent increase in Fig. 1 was not due to volume itself but was more likely due to concentration or amount of Na⁺ delivered to the receptor. In the high-NaCl and high-KCl diet groups, the dose-response curves were significantly attenuated compared with the normal diet group [0.375 M NaCl, F(2,117) = 5.665, P = 0.0045; 0.75 M NaCl, F(2,117) = 9.123, P = 0.002; 1.5 M NaCl, F(2,117) = 12.626, P < 0.0001, respectively].

Figure 2A shows typical HANA responses to intraportal injection of 50 mM KCl + 100 mM NaCl (0.5 ml/kg body wt) in rats on the normal, high-NaCl, or high-KCl diets. The responses were similar to those seen after injection of 0.75 M NaCl solution. The summarized data for the peak responses to
different volumes of three isotonic KCl + NaCl solutions are presented in Fig. 2, B–D. As with the hypertonic NaCl solution, intraportal injection of isotonic KCl + NaCl solution resulted in a K⁺ dose-dependent increase in HANA in the normal diet group, while the responses in the high-NaCl and high-KCl diet groups were significantly reduced [25 mM KCl + 125 mM NaCl, F(2,117) = 8.108, P = 0.0005; 50 mM KCl + 100 mM NaCl, F(2,117) = 5.599, P = 0.0048; 100 mM KCl + 50 mM NaCl, F(2,117) = 11.031, P < 0.0001, respectively]..

**RT-PCR.** Figure 3A shows the electrophoretic separation of the RT-PCR products for NKCC1 and cyclophilin. The NKCC1 transcript was seen as a 425-bp band. In both the high-NaCl and high-KCl diet groups, the NKCC1 transcript was expressed at a lower level than in the normal diet group, whereas similar expression of the cyclophilin transcript was seen in all three groups. Figure 3B shows the NKCC1/cyclophilin density ratio for the RT-PCR products in the three groups. In both the high-NaCl and high-KCl diet groups, NKCC1 mRNA expression was significantly reduced compared with in animals on the normal diet [F(2,21) = 6.244, P = 0.0074].

**Western blots.** Figure 3C shows Western blots using T4 anti-NKCC1 antibody. A protein band of 140 kDa was detected. In both the high-NaCl and high-KCl diet groups, expression of NKCC1 protein was much lower than in the normal diet group. Figure 3D shows the quantitative results for NKCC1 protein expression in the three groups. In both the high-NaCl and high-KCl diet groups, NKCC1 protein expression was significantly decreased compared with that in the normal diet group [F(2,21) = 13.40, P = 0.0002].

**DISCUSSION**

The major findings of the present study are as follows. 1) Administration of a high oral NaCl or KCl load for 4 wk reduced hepatic Na⁺ and K⁺ receptor sensitivity, i.e., the increase in HANA in response to intraportal injection of hypertonic NaCl and isotonic KCl + NaCl was attenuated by a high-NaCl or high-KCl diet. 2) The level of NKCC1 mRNA and protein expression was also reduced by a high-NaCl or high-KCl diet.

It is well known that the amount of a given substance in the diet can alter the activity and expression of the transport system responsible for the transport of that substance (13, 20, 26, 27). A low-NaCl diet upregulates the amiloride-sensitive Na⁺ channel in the lower intestine, the distal convoluted tubule, and the medullary collecting duct of the kidney, while a high-Na⁺ diet downregulates the channel (13, 27). These changes are thought to be an adaptation to low- and high-Na⁺ environments because changes in expression of the amiloride-sensitive Na⁺ channel in the intestine or the kidney affect, respectively, Na⁺ absorption or excretion. However, the NKCC1 downregulation seen in the present study does not seem to be involved in maintaining Na⁺ and K⁺ homeostasis on a high-NaCl and high-KCl diet.

A previous study from our laboratory demonstrated that NKCC1 plays a major role in transducing intraportal Na⁺ and K⁺ concentrations into hepatic afferent nerve activity (16, 17). Thus, if NKCC1 was downregulated, the sensitivity of hepatic Na⁺ and K⁺ receptors should be reduced, which was the case in the present study. Because hepatic Na⁺ and K⁺ receptors play a significant role in postprandial natriuresis and kaliuresis,
reduced sensitivity of hepatic Na\(^+\) and K\(^+\) receptors may not affect the long-term regulation of Na\(^+\) and K\(^+\) homeostasis. Another possible explanation is that the decreased hepatic Na\(^+\) and K\(^+\) receptor sensitivity reduces the role of these receptors in the long-term control of Na\(^+\) and K\(^+\) homeostasis.

In addition to Na\(^+\) and K\(^+\) sensor functions, NKCC1 in the liver is known to regulate the volume of hepatocytes. In cultured hepatocytes and perfused rat liver, hypertonic stress induces a regulatory cell volume increase, in which the Na\(^+\) channel, Na\(^+\)/H\(^+\) exchanger, and NKCC1 play a significant role in Na\(^+\) influx (7, 29). In the present study, NKCC1 was downregulated after 4 wk on a high-NaCl or high-KCl diet, suggesting that Na\(^+\) influx into hepatocytes might decrease. However, Li et al. (12) reported that the Na\(^+\)-K\(^+\)-ATPase activity of the liver is significantly higher in rats that have received a 3-mo oral load of 1.8% NaCl than in control rats. Their result suggests that Na\(^+\) influx into hepatocytes might be increased on a high-NaCl diet compared with a normal diet because the total Na\(^+\) influx is equal to the amount of Na\(^+\) efflux via Na\(^+\)-K\(^+\)-ATPase plus the observed increase in intracellular Na\(^+\) (29). To understand this discrepancy, it should be noted that the contribution of NKCC1 to Na\(^+\) influx during the regulatory volume increase is relatively small. Wehner and Tinel (29) estimated that the Na\(^+\) channel, Na\(^+\)/H\(^+\) exchanges, and NKCC1 contribute to Na\(^+\) influx in the ratio of 4:1:1. Thus it is possible that the Na\(^+\) channel and Na\(^+\)/H\(^+\) exchanger are the main contributors to the increased Na\(^+\) influx during long-term high NaCl load. If this is the case, downregulation of NKCC1 might be an adaptive phenomenon to prevent excessive Na\(^+\) accumulation in the hepatocyte.

The mechanism linking a high-NaCl or high-KCl diet to decreased NKCC1 expression is not clear. Plasma levels of vasopressin and aldosterone are influenced by the Na\(^+\) and K\(^+\) content of the diet, and these hormones are known to regulate the expression of ion transporters (10, 13). In the present study, both a high-NaCl diet and a high-KCl diet reduced NKCC1 expression in the liver. Because high-NaCl and high-KCl diets have opposite effects on plasma aldosterone levels (6), aldosterone might not be involved in the reduction in NKCC1 expression. On the other hand, both a high-NaCl and a high-KCl diet increase plasma vasopressin levels (3, 11), which causes an increase in the expression of NKCC2 (responsible for absorption) in the kidney (10). Thus it is possible that vasopressin might be involved in the mechanism of NKCC1 downregulation in the liver. Another possibility is that hypertonicity itself modifies NKCC1 expression. Anzai et al. (2) demonstrated that hypertonicity increased NKCC1 mRNA expression in cultured kidney inner medullary collecting duct cells, so hypertonicity might alter NKCC1 expression in the liver in animals on either a high-NaCl diet or a high-KCl diet. Actually, Schliess et al. (25) demonstrated that hypotonic exposure of HuH7 human hepatoma cells by elevation of extracellular NaCl induces an increase in NKCC1 mRNA expression, which was maximal between 8 and 16 h and declines thereafter. However, the effect on NKCC1 expression in the cultured hepatocyte in their study was the opposite of that seen in the liver in the present study. These differences necessitate further studies.

In conclusion, a long-term high-NaCl or high-KCl diet causes a decrease in hepatic Na\(^+\) and K\(^+\)-receptor sensitivity, which might be due to the associated reduction in NKCC1 expression seen in the liver.

**Perspectives**

NKCC1 is known to exist in both the liver parenchymal cells and nonparenchymal cells (25). Furthermore, the existence of NKCC1 is demonstrated in the cell bodies and dendrites of neurons in the central and peripheral nervous system (24). Although the site of existence of NKCC1, which is responsible for the hepatic Na\(^+\) and K\(^+\) sensor mechanism, is unknown, two possibilities can be considered. First, the hepatic nerve terminal might be in contact with hepatocytes that bear NKCC1. Second, the nerve terminal itself might bear NKCC1. In the present study, we did not determine where the decrease in NKCC1 expression occurred. Thus whether the decrease in NKCC1 observed in the present study is due to the decrease in NKCC1 that is responsible for the sensor mechanism is unclear. However, at least functional evidence that demonstrates the decreased sensor mechanism was provided by the present study. To further understand the physiological and/or pathophysiological significance of the decreased hepatic NKCC1, the linkage between NKCC1 and hepatic afferent nerve and the cell type that is responsible for the decreased NKCC1 have to be examined in future studies.

**GRANTS**

This study was partly supported by research grants from the Ministry of Education, Science, and Culture of Japan and Salt Science Japan.

**REFERENCES**


