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Expression of three isoforms of Na-K-2Cl cotransporter (NKCC2) in the kidney and regulation by dehydration

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ABSTRACT

Sodium reabsorption via Na-K-2Cl cotransporter 2 (NKCC2) in the thick ascending limbs has a major role for medullary osmotic gradient and subsequent water reabsorption in the collecting ducts. We investigated intrarenal localization of three isoforms of NKCC2 mRNA expressions and the effects of dehydration on them in rats. To further examine the mechanisms of dehydration, the effects of hyperosmolality on NKCC2 mRNA expression in microdissected renal tubules was studied. RT-PCR and RT-competitive PCR were employed. The expressions of NKCC2a and b mRNA were observed in the cortical thick ascending limbs (CAL) and the distal convoluted tubules (DCT) but not in the medullary thick ascending limbs (MAL), whereas NKCC2f mRNA expression was seen in MAL and CAL. Two-day dehydration did not affect these mRNA expressions. In contrast, hyperosmolality increased NKCC2 mRNA expression in MAL *in vitro*. Bradykinin dose-dependently decreased NKCC2 mRNA expression in MAL. However, dehydration did not change NKCC2 protein expression in membrane fraction from cortex and outer medulla and in microdissected MAL.

These data show that NKCC2a/b and f types are mainly present in CAL and MAL, respectively. Although NKCC2 mRNA expression was stimulated by hyperosmolality *in vitro*, NKCC2 mRNA and protein expressions were not stimulated by dehydration *in vivo*. These data suggest the presence of the inhibitory factors for NKCC2 expression in dehydration. Considering the role of NKCC2 for the countercurrent multiplier system, NKCC2f expressed in MAL might be more important than NKCC2a/b.

1. Introduction

Sodium and water reabsorption by the kidney has a major role for the maintenance of body fluid homeostasis [1]. Sodium reabsorption in the thick ascending limbs produces interstitial hypertonicity in medulla and induces water reabsorption in the collecting ducts through vasopressin-dependent water channel, aquaporin 2 (AQP2) [2,3]. Na-K-2Cl cotransporter 2 (NKCC2) has a major role for sodium reabsorption in the distal nephron as well as thiazide-sensitive Na-Cl cotransporter in the distal convoluted tubules and amiloride-sensitive epithelial sodium channel (ENaC) in the collecting ducts [3]. NKCC2 is present in the medullary and cortical thick ascending limbs (MAL and CAL, respectively) and is sensitive to furosemide, a most potent diuretic [4–6]. Three types of isoforms of NKCC2 have been reported; a, b, and f types [7–12]. The F type has been reported in the outer medullary collecting ducts (OMCD), which was not confirmed by immunohistochemistry.

Water reabsorption through AQP2 is sensitive to vasopressin. Plasma level of vasopressin is known to increase in dehydration. Since sodium reabsorption in the thick ascending limbs has a major

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role for medullary osmotic gradient, which is a driving force for water reabsorption in the collecting ducts, NKCC2 has been thought to be activated by dehydration [13]. Ecelbarger et al. reported the lack of stimulation of NKCC2 expression by dehydration [4]. However, they reported that long-term AVP infusion to Brattleboro rats stimulated NKCC2 expression [14]. Although the increase in NaCl reabsorption via NKCC2 has been speculated as a source of increased medullary osmotic gradient in dehydration, it seems that NKCC2 expression is not regulated by dehydration [13].

Bradykinin is known to regulate Na reabsorption in the cortical collecting ducts (CCD) [15]. Angiotensin-converting enzyme inhibitor is known to cause renoprotective effects by reducing blood pressure and proteinuria and a part of the effects is brought by the decrease in breakdown of bradykinin [16]. However, no report has examined the effects of bradykinin on sodium transport in MAL.

The aim of our study was to know precise localization of mRNA expression of NKCC2 three isoforms, and to determine whether NKCC2 mRNA and protein expressions are regulated by either osmotic change or dehydration.

2. Methods

2.1. Animals

Four-week-old male SD rats were used in this study. Rats were given standard rat chow. Dehydrated rats were deprived tap water for two days. Serum and urine osmolality of the rats were measured using micro-osmometer (Fiske, MA, USA) with sample volume of 15 μl. The protocol of our study was checked and approved by the committee on animal care in the Kumamoto University Graduate School of Medical Sciences (18-127, 19-063) and Kitasato University Medical Center (25-2).

2.2. Microdissection of nephron segments

Microdissection of nephron segments was performed as previously reported [17,18]. Following nephron segments were microdissected; glomerulus (Glm), proximal convoluted and straight tubule (PCT and PST, respectively), medullary and cortical thick ascending limb (MAL and CAL, respectively), distal convoluted tubule (DCT), cortical, outer medullary and inner medullary collecting ducts (CCD, OMCD, and IMCD, respectively). 2-mm long dissected tubules were used for RT-PCR and RT-competitive PCR.

2.3. Incubation study

To examine the effects of hyperosmolality on NKCC2 mRNA expression, microdissected MAL was incubated in hypertonic medium for 1 h at 37 °C. Hypertonic solution was made by the addition of NaCl to the isotonic medium [18]. Bradykinin was also used for the incubation study to examine the effects of bradykinin on NKCC2 mRNA expression. Microdissected MAL was incubated in isotonic medium with bradykinin or vehicle for 1 h at 37 °C. After the incubation, incubated MAL was used for RT-competitive PCR.

2.4. RT-PCR and RT-competitive PCR

Conventional RT-PCR was performed to examine the mRNA localization of NKCC2 along the nephron in control and dehydrated rats as described previously [19].

The sense primer for NKCC2a, b, and f isoforms are modified with bases 863–884 of murine isoforms [8] (U20973-5): -a: (5′-AATTCTTCTTTCCACCATGGTA-3′, bases 832-853 in EF577032), -b: (5′-CATCGGCTTAGCCGTGACAGTG-3′), and -f: (5′-CATTGGCCT GAGCGTGGTAGTG-3′, bases 848-869 in NM_001270618, underlined bases were changed from murine). The antisense primer for NKCC2a, b, and f isoforms are same and defined by bases 1460-1481 (U20973-5) (bases 1429-1450 in EF577032, bases 1445-1466 in NM_001270618) (5′-GATGAAAATGGCCAGCATGG TC-3′). The size of PCR product was 619 bp in three isoforms.

To examine precise changes of the expression of NKCC2 mRNA, microdissected MAL, CAL and DCT was taken for quantitative RT-competitive PCR. In addition, MAL was incubated in hypertonic medium to examine the osmotic effect on the expression of NKCC2 mRNA. To perform competitive PCR, the DNA competitor was synthesized using overlap-extension PCR as previously described [17]. NKCC2 inner sense and antisense primers were defined by bases 1114-1134 (NM_001270618) (5′-AACGGAGTGGTGAGAGGAGGTT CGGTTCCATCACGGTGGTGA-3′) and 909-929 (NM_001270618) (5′-TCACCACCGTGATGGAACCGAACCTCCACGAACAAACCCGTT-3′), respectively (the complementary sequence added to the 5′ tail is underlined). The size of PCR products of NKCC2 mRNA and DNA competitor was 619 and 394 bp, respectively.

The sense and antisense primers for NKCC2 (common ones for a, b, and f) were defined by bases 1379-1398 (5′-TGGGATTCTTGCT GGTGCCA-3′) and 1926-1945 (5′-AGCGCATATGAGGCCAGGAA-3′) (NM_001270618). The inner-sense and inner-antisense primers for DNA competitor were defined by bases 1713-1730 (5′-GACTGG GCTACGACTTCTACACTGTCTTCGGCCCTA-3′) and 1588-1605 (5′-TA GGGCCGAAGACAGTGTAGAAGTCGTAGCCCAGTC-3′) (NM_0012706 18), respectively (the complementary sequence added to the 5′ tail is underlined). The size of PCR products of NKCC2 mRNA and DNA competitor was 567 and 425 bp, respectively.

2.5. Western blot analysis

Western blot was performed using the membrane fraction of renal cortex, outer medulla, and inner medulla, and microdissected OMCD and MAL as described previously [17,18].

After SDS–PAGE, the protein was transferred to nylon membrane. The membrane was incubated with the primary antibody against NKCC2 (kind gift from Dr. S.C. Hebert) over night. Then, the membrane was incubated with horseradish peroxidase-linked anti-rabbit IgG F(ab')2 for 1 h at room temperature. The protein expression was visualized with ECL Select Western blotting detection reagents (GE Healthcare Japan, Hino, Tokyo).

2.6. Statistical analyses

Data are expressed as mean ± SEM. The differences in the value were examined with either Student's *t*-test or analysis of variance (ANOVA) and multiple comparison of Dunnet's type. A $p < 0.05$ was considered as significant.

3. Results

3.1. mRNA expression of three isoforms of NKCC2 along the nephron

At first, mRNA expression of three isoforms was examined using RT-PCR. In control rats, NKCC2a and NKCC2b mRNAs were expressed in CAL and DCT (CAL > DCT) but not in MAL (Fig. 1A and B). NKCC2f mRNA was expressed in mainly in MAL and slightly in CAL (Fig. 1C). No expression of three isoforms was observed in OMCD in control rats.

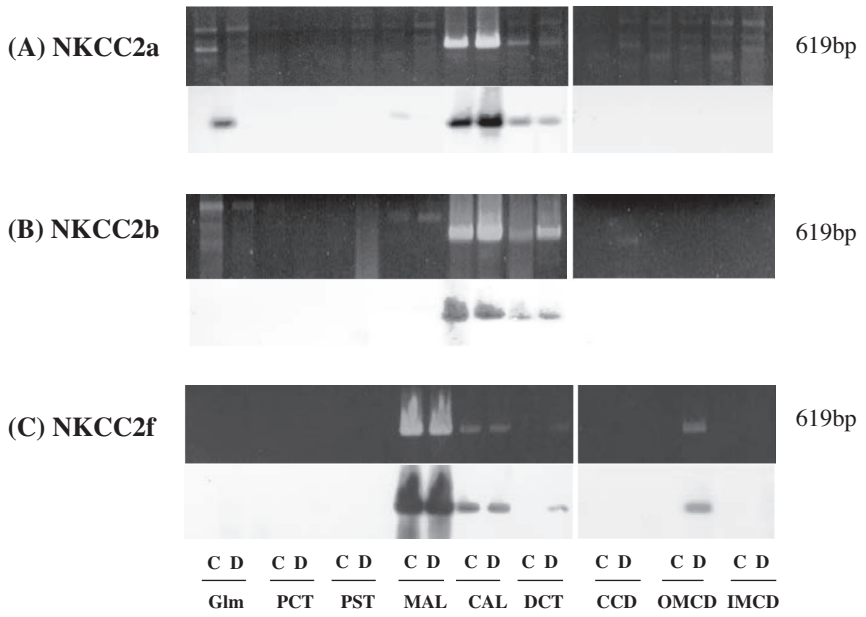

Fig. 1. NKCC2a (A), NKCC2b (B) and NKCC2f (C) mRNA expressions along the nephron in control and dehydrated rats. Three isoforms of NKCC2 mRNA, a, b and f, were examined using RT-PCR. Upper parts show the typical gel of RT-PCR and lower parts represent corresponding Southern blot. (A) NKCC2a mRNA was localized in CAL and DCT, and dehydration did not change the abundance. (B) NKCC2b mRNA was present in CAL and DCT, and dehydration did not influence the expression. (C) NKCC2f mRNA was identified in MAL and CAL, and dehydration did not influence the expression in MAL and CAL. However, dehydration caused the expression of NKCC2f mRNA in DCT and OMCD, where no signal was observed in control rats.

3.2. Effect of dehydration on mRNA expression of three isoforms

The expression of NKCC2f mRNA was stimulated in DCT and OMCD by two days dehydration, while no expression was observed in the same segments in control rats (Fig. 1C). The expression of NKCC2b mRNA in DCT was also stimulated by the dehydration (Fig. 1B). In contrast, mRNA expressions of all three isoforms in MAL and CAL were not changed by dehydration (Fig. 1A–C). Competitive RT-PCR was used for precise evaluation of mRNA changes by dehydration. mRNA expressions of NKCC2a and NKCC2b in CAL and NKCC2f in MAL was not changed by dehydration (Fig. 2A–C, $n = 3–5$).

3.3. Effect of hyperosmolality and bradykinin on NKCC2 mRNA expression

To know the mechanisms of regulation of NKCC2 stimulation, effects of hyperosmolality on NKCC2 mRNA expression was examined *in vitro* by RT-competitive PCR. Incubation of microdissected MAL in hypertonic solution (>690 mOsm/kgH_2O) by NaCl for 1 h increased NKCC2 mRNA expression (Fig. 3A, $n = 4$).

Next, effect of bradykinin on NKCC2 mRNA expression in MAL was studied. Bradykinin dose-dependently inhibited NKCC2 mRNA expression in MAL (Fig. 3B, $n = 4$).

3.4. Effect of dehydration on NKCC2 protein expression

Effect of dehydration on NKCC2 protein expression was examined using membrane fraction of the kidney and microdissected nephron segments. NKCC2 protein was detected in the membrane fraction from cortex and outer medulla, and in MAL but not in OMCD at 160–170 kDa (Fig. 4A and B, $n = 3$). Two-days dehydration, however, did not change the expressions in them.

4. Discussion

Our study showed that (1) in control rats, NKCC2a and NKCC2b mRNAs were expressed in CAL and DCT (CAL > DCT). NKCC2f mRNA was expressed in MAL and CAL (MAL > CAL). No expression of f was observed in OMCD. Although two-days dehydration stimulated NKCC2f mRNA expression in DCT and OMCD, mRNA expressions of three isoforms in MAL and CAL were not changed by dehydration. (2) Incubation of MAL in hypertonic solution (NaCl added) for 1 h increased NKCC2 mRNA expression. (3) NKCC2 protein was detected in the membrane fraction from cortex and outer medulla, and in microdissected MAL but not in OMCD at 160–170 kDa. Dehydration did not change the expressions. (4) Bradykinin dose-dependently decreased NKCC2 mRNA expression in MAL.

Intrarenal localization of three isoforms of NKCC2 mRNA was different. NKCC2a and NKCC2b mRNA expressions were observed in CAL and DCT (CAL > DCT). In contrast, NKCC2f mRNA expression was seen in MAL and CAL (MAL > CAL). Therefore, NKCC2a/b and NKCC2f represent NKCC2 in CAL and MAL, respectively. Although our results are slightly different from those by Igarashi et al., our methods are useful as well as in situ hybridization performed by them [8]. NaCl reabsorption by NKCC2 is a source of medullary osmotic gradient and subsequent reabsorption of water through AQP2 in the collecting ducts [1–3,13]. NKCC2 protein expression was observed in MAL but not in OMCD. NKCC2 expression in MAL is more important than that in CAL to form medullary osmotic gradient. These data suggest that NKCC2f isoform may be more important than the other two isoforms. Although low NKCC2f mRNA expression was observed in OMCD from dehydrated rats, no protein abundance was seen in microdissected OMCD both in control and dehydration, supposing no functional role of NKCC2f in OMCD.

Since water reabsorption by the kidney is increased in dehydration and NaCl reabsorption through NKCC2 in MAL is a source of medullary hypertonicity, upregulation of NKCC2 by dehydration has been expected. However, the data so far indicated the lack of

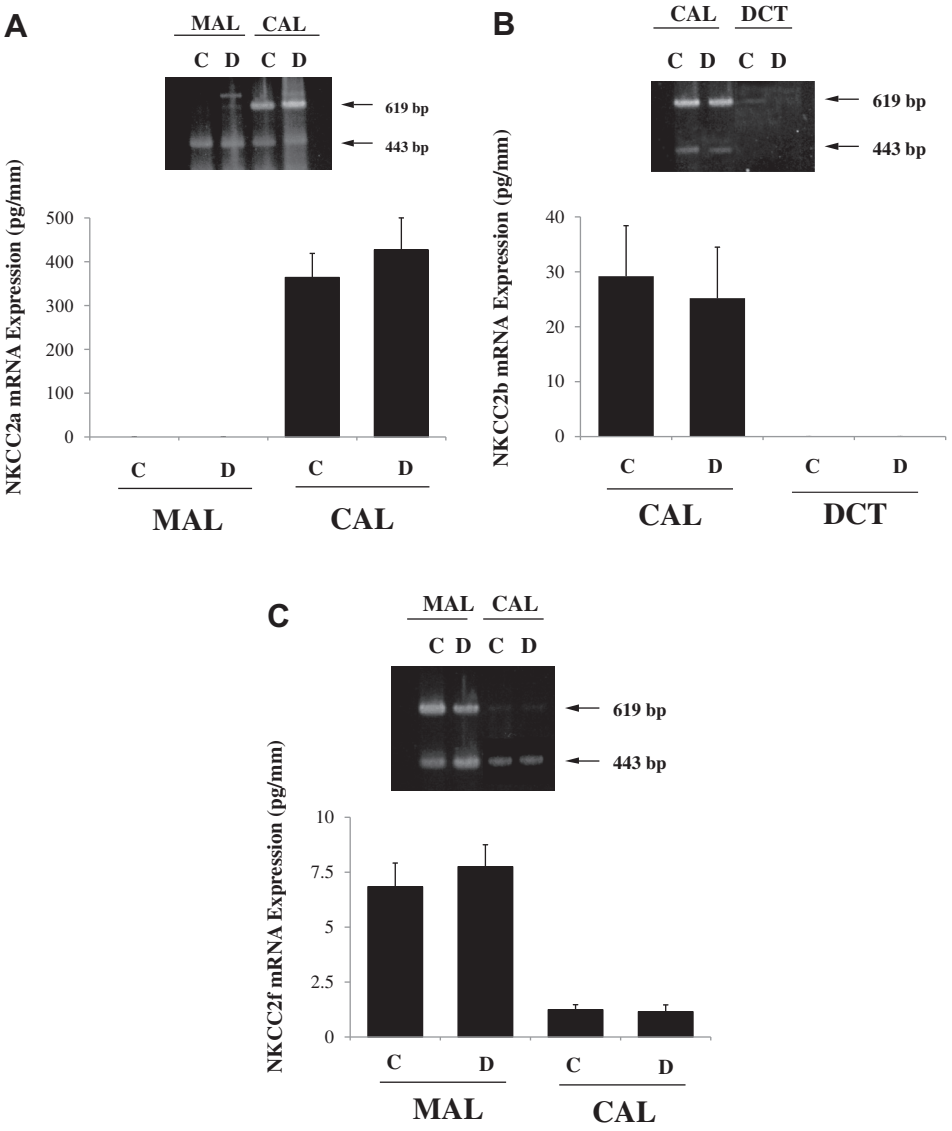

Fig. 2. Quantitative changes of NKCC2a (A), NKCC2b (B), and NKCC2f (C) mRNA in MAL, CAL, and DCT by dehydration. NKCC2 isoform expressions in control and dehydration were examined using competitive PCR. (A) NKCC2a mRNA expression in CAL was not changed by dehydration. The expression was not observed in MAL by competitive PCR. (B) NKCC2b mRNA expression in CAL and DCT was not influenced by dehydration. (C) NKCC2f mRNA expression was observed in MAL and CAL by quantitative PCR. The abundance was larger in MAL than in CAL. Those were not changed by dehydration $n = 3–5$.

stimulation of NKCC2 expression by dehydration. Dehydration is known to increase plasma level of vasopressin and plasma and urine osmolality. Ecellbarger et al. showed that arginine vasopressin (AVP) infusion increased NKCC2 expression in Brattleboro rats but not in control rats [4,14]. Our data show that hyperosmolality increased NKCC2 mRNA expression in MAL. Salt intake has been reported to influence the activity of isoforms [20]. These data suggest that dehydration should increase NKCC2 expression in MAL via increased plasma AVP level and/or hyperosmolality of plasma and urine. The presence of inhibitory factor such as SPAK isoforms is speculated [21,22]. We show that increased PGE2 production in dehydration caused downregulation of vasopressin V2 receptor (V2R) expression in the inner medullary collecting ducts. Similar mechanism of inhibition of NKCC2 by PGE2 is possible as a reason of the lack of upregulation of NKCC2 by dehydration. The role of three isoforms of NKCC2 on sodium transport has to be examined further [23]. Using isolated perfusion of MAL, we showed that AVP stimulated chloride absorption in long-looped MAL but not short-looped MAL, suggesting different localization of V2R and V1aR or NKCC2 isoforms in long- and short-looped MAL [24]. We also showed that V2R and V1aR are present and AVP inhibits HCO_3^- reabsorption both in long- and short-looped MAL [25]. So far, it is not known why such a difference in AVP-stimulated Cl absorption is present in long- and short-looped MAL. Localization of three isoforms in long- and short-looped MAL has to be examined.

Bradykinin is known to regulate sodium reabsorption in CCD [15]. A part of the renoprotective effects of angiotensin-converting enzyme inhibitor has been thought to be driven by the accumulation of bradykinin. Our data showed that bradykinin dose-dependently inhibited NKCC2 mRNA expression in MAL. It could be possible that bradykinin inhibited sodium reabsorption via NKCC2. One of the problem for the physiological role of bradykinin on NKCC2 function is that high doses of bradykinin were required for the inhibition of NKCC2 mRNA, Low dose of bradykinin (10^{-7} M) inhibited vasopressin-stimulated NaCl reabsorption in CCD from DOCA-treated rats [15]. Such a shift of dose response curve to the right was observed also in AVP-stimulated cAMP generation [26]. It must be examined whether low-doses of bradykinin influences NaCl reabsorption in MAL. It is not known whether intrarenal level of bradykinin is increased in dehydration. If plasma

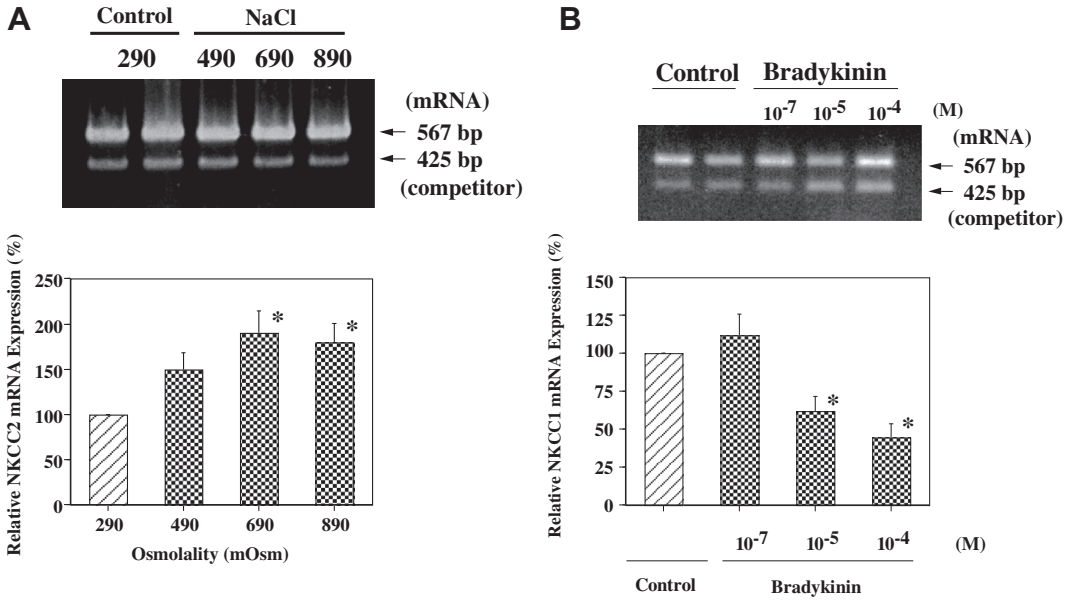

Fig. 3. Effect of hyperosmolality and bradykinin on NKCC2 mRNA expression in MAL *in vitro*. Effect of hyperosmolality and bradykinin on NKCC2 mRNA expression in MAL was examined. Microdissected MAL was incubated in isotonic or hypertonic medium. Hypertonic solution was made by the addition of NaCl to the isotonic solution. (A) Hyperosmolality of more than 690 mOsm/KgH_2O caused the increase of NKCC2 mRNA expression. $^*p < 0.05$ vs. 290 mOsm/KgH_2O. ($n = 4$). (B) In contrast, high doses of bradykinin dose-dependently inhibited NKCC2 mRNA expression. $^*p < 0.05$ vs. control. ($n = 4$).

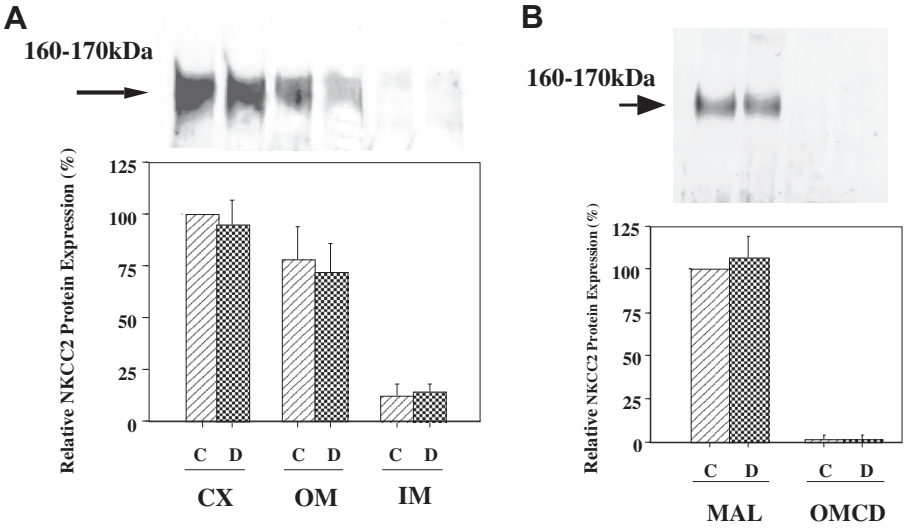

Fig. 4. Effect of dehydration on NKCC2 protein expression in membrane fraction and microdissected MAL and OMCD. Membrane fraction of cortex, outer medulla, and inner medulla and microdissected MAL and OMCD from control and dehydrated rats were used for Western blotting. NKCC2 expression was observed in membrane fraction of each part (cortex > outer medulla > inner medulla) and in MAL but not in OMCD. Dehydration did not change the expression of NKCC2 in membrane fractions and in MAL ($n = 3$).

or intrarenal levels of bradykinin are increased in dehydration, NKCC2 expression could be inhibited. Bradykinin has a possibility as an inhibitory factor of NKCC2 expression *in vivo* in dehydration.

In summary, NKCC2a/b and f type mRNA expressions were observed in CAL and MAL, respectively. Although NKCC2 mRNA expression is stimulated by hyperosmolality, NKCC2 protein expression was not stimulated by dehydration. Considering the increase in medullary osmotic gradient in dehydration, the presence of inhibitory factors in dehydration, such as PGE2 or bradykinin, is speculated. Considering the role of NKCC2 for the countercurrent multiplier system, NKCC2f expressed in MAL might be more important than NKCC2a/b. Further studies are required to elucidate the role of NKCC2 for medullary osmotic gradient especially in dehydration.

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