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Differential regulation of Na^+,K^+-ATPase and the Na^+-coupled glucose transporter in hypertensive rat kidney

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Abstract

Several Na^+ transporters are functionally abnormal in the hypertensive rat. Here, we examined the effects of a high-salt load on renal Na^+,K^+-ATPase and the sodium-coupled glucose transporter (SGLT1) in Dahl salt-resistant (DR) and salt-sensitive (DS) rats. The protein levels of Na^+,K^+-ATPase and SGLT1 in the DS rat were the same as those in the DR rat, and were not affected by the high-salt load. In the DS rat, a high-salt load decreased Na^+,K^+-ATPase activity, and this decrease coincided with a decrease in the apparent Mechaelis constant (K_m) for ATP, but not with a change of maximum velocity (V_{max}). On the contrary, a high-salt load increased SGLT1 activity in the DS rat, which coincided with an increase in the V_{max} for α-methyl glucopyranoside. The protein level of phosphorylated tyrosine residues in Na^+,K^+-ATPase was decreased by the high-salt load in the DS rat. The amount of phosphorylated serine was not affected by the high-salt load in DR rats, and could not be detected in DS rats. On the other hand, the amount of phosphorylated serine residues in SGLT1 was increased by the high-salt load. However, the phosphorylated tyrosine was the same for all samples. Therefore, we concluded that the high-salt load changes the protein kinase levels in DS rats, and that the regulation of Na^+,K^+-ATPase and SGLT1 activity occurs via protein phosphorylation.

Keywords: Hypertension; SGLT1; Na^+,K^+-ATPase; Dahl rat; Phosphorylation; Kidney

1. Introduction

Na^+,K^+-ATPase of renal epithelial cells plays a very important role in the regulation of Na^+ balance, extracellular volume and blood pressure. The function and regulation of renal Na^+,K^+-ATPase are changed in the pathogenesis of hypertension [1,2]. Hypertensive Dahl salt-sensitive (DS) rats, an animal model for genetic hypertension, and Dahl salt-resistant (DR) rats have been frequently used to determine functional changes of ion channels, pumps and transporters. In particular, it has been reported that Na^+ uptake was affected by a high-salt load in DS rat, and the mechanism involved in the regulation may include the Na^+ channel [3], Na^+/H^+-antiporter [4,5], Na^+/Ca^{2+}-exchanger [6] and Na^+,K^+-ATPase [7]. However, the regulatory factors were examined in detail.

Protein kinase mediated phosphorylation is involved in the regulation of Na^+,K^+-ATPase. Both protein kinase C and A can phosphorylate the Na^+,K^+-ATPase α subunit in DS rat kidney [2],

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and in addition the protein kinase C mediated phosphorylation was associated with an inhibition of enzyme activity [8]. The affinity of Na^+,K^+-ATPase for K^+ was increased in cortical tubule cells of normal-salt loaded DS rat, but such an effect was not observed in basolateral membrane vesicles or the purified enzyme [2].

The Na^+-coupled glucose transporter (SGLT1) is expressed in intestinal and renal epithelial cells [9,10]. This transporter is responsible for the absorption of glucose. The uphill transport of sugar is coupled to Na^+ transport down its electrochemical potential gradient formed by Na^+,K^+-ATPase. Therefore, it is necessary to investigate the functional changes of both Na^+,K^+-ATPase and SGLT1 together. SGLT1 was directly activated by protein kinase A-mediated phosphorylation in rat small intestine [11]. Vazquez et al. [12] reported that SGLT1 activity was increased in spontaneously hypertensive rats.

In the present study, we investigated whether the protein level of Na^+,K^+-ATPase and SGLT1 was affected by a high-salt load in DR and DS rats. We also examined the relationship between the activities of Na^+,K^+-ATPase and SGLT1 and their protein phosphorylation levels.

2. Materials and methods

2.1. Isolation of renal basolateral membrane vesicle (BLMV) and brush border membrane vesicle (BBMV)

BLMV and BBMV of kidney were prepared from male DR and DS rats as described previously [13] with minor modifications. DR and DS rats were divided into two groups, and from weaning at 15 days of age each group had free access to high-salt (8% NaCl) or normal-salt (0.2% NaCl) diets. All rats were anesthetized with ether and decapitated within 15 days of salt feeding. The kidneys were removed and rinsed with phosphate-buffered saline. BLMV and BBMV were prepared using the Mg^{2+}–EGTA precipitation method. The isolated vesicles were stored at −85°C until use. Protein concentrations were measured using the protein assay kit (Bio-Rad Laboratories, CA, USA) with bovine serum albumin as the standard.

2.2. Assay of Na^+,K^+-ATPase activity

Na^+,K^+-ATPase activity of renal BLMV was assayed in 1 ml of a solution containing 10 μg of membrane protein, 3 mM $MgCl_2$, 1 mM ATP, 5 mM NaN_3, 120 mM NaCl, 15 mM KCl and 40 mM Tris–HCl (pH 7.4), in the presence or absence of ouabain (1 mM). After incubation for 30 min at 37°C, the reaction was terminated by the addition of ice-cold stop solution containing 12% perchloric acid and 3.6% ammonium molybdate. The inorganic phosphate released was measured from the absorbance at the wavelength of 320 nm as described elsewhere [14]. Net Na^+,K^+-ATPase activity was calculated as the difference between the ATPase activities in the presence and absence of ouabain.

2.3. Assay of SGLT1 activity

SGLT1 activity of renal BBMV was measured by the method of Inui et al. [15]. The transporter activity was assayed by incubating the BBMV in a reaction solution containing 1 mM [α-^{14}C]methyl glucopyranoside (AMG, 50 μCi/ml), 50 mM NaCl, 100 mM mannitol, and 20 mM Hepes–Tris (pH 7.5), in the presence or absence of phloridzin (0.1 mM), a potent SGLT1 inhibitor. After incubation for 2 min at 25°C, the reaction was terminated by the addition of ice-cold stop solution containing 150 mM NaCl, 0.1 mM phloridzin, and 20 mM Hepes–Tris, pH 7.5. Aliquots were rapidly filtered through 0.45-μm millipore filters, and then washed with 5 ml of ice-cold stop solution. Net SGLT1 activity was calculated as the difference between the activities in the presence and absence of phloridzin.

2.4. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and immunoblotting

SDS–polyacrylamide gel electrophoresis was carried out as described elsewhere [16] with minor modifications. Membrane preparations (20 or 40 μg) were boiled in a sample buffer containing 1.1% SDS, 2.5% sucrose, 0.001% bromophenol blue, 2.5% 2-mercaptoethanol, and 30 mM Tris–HCl, pH 6.8 for 3 min and then applied to the SDS–polyacrylamide gel. Proteins were blotted onto a PVDF membrane in

transfer solution containing 20% methanol, 40 mM glycine, and 25 mM Tris–HCl, pH 9.4. The membranes were soaked in a blocking solution containing 75 mM NaCl, 2% skimmed milk, and 25 mM Tris–HCl, pH 7.2 for 6 h. The membranes were then incubated for 3 h with each primary antibody diluted in blocking solution. The blots were washed in the blocking solution supplemented with 0.1% Tween-20 and incubated with peroxidase-conjugated anti-rabbit IgG for 1 h. Finally, the blots were stained with the ECL Western blotting kit from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

2.5. Antibody

The Na^+,K^+-ATPase α subunit specific polyclonal antibody was previously raised against the amino-terminal peptide (residues 1–92). The anti-phosphotyrosine antibody was previously raised against hybridoma G8.D6 strain in our laboratory. The anti-phosphoserine antibody was purchased from Sigma, and the rabbit anti-SGLT1 antibody from Chemical International (Temecula, USA).

2.6. Immunoprecipitation

Membrane fractions of BLMV and BBMV were incubated in lysis buffer containing 1% Nonidet P-40, 150 mM NaCl, 0.5 mM EDTA, and 50 mM Tris–HCl (pH 7.4), at 4°C for 30 min. After centrifugation at $16\,000 \times g$ for 5 min, the supernatant was incubated with Protein G Sepharose (Amersham Pharmacia Biotech) and an antibody specific for the Na^+,K^+-ATPase α subunit or SGLT1 at 4°C for 6 h. After centrifugation, the pellet was washed four times with lysis buffer followed by two washes in 0.1% Nonidet P-40, 150 mM NaCl, 0.5 mM EDTA, and 50 mM Tris–HCl, pH 7.4. The pellet was solubilized in the sample buffer for SDS–polyacrylamide gel electrophoresis. The immunoblotting was carried out as described in Section 2.4.

2.7. Statistics

Results are presented as the mean ± S.E.M. Differences between the groups were analyzed by one-way analysis of variance (ANOVA), and correction for multiple comparisons was made by using Turkey's multiple comparison test. Statistically significant differences were assumed at $P < 0.05$.

3. Results

3.1. Immunoblot analysis of BLMV and BBMV proteins

Na^+,K^+-ATPase was expressed in the renal basolateral membrane. The α subunit is a 100 kDa polypeptide. We compared the protein amount of Na^+,K^+-ATPase α subunit protein in the high- and normal-salt groups using BLMV (Fig. 1A). The expression of Na^+,K^+-ATPase in the high-salt groups are same as that in normal-salt groups, and DS rats were same as DR rats. Next, we compared the

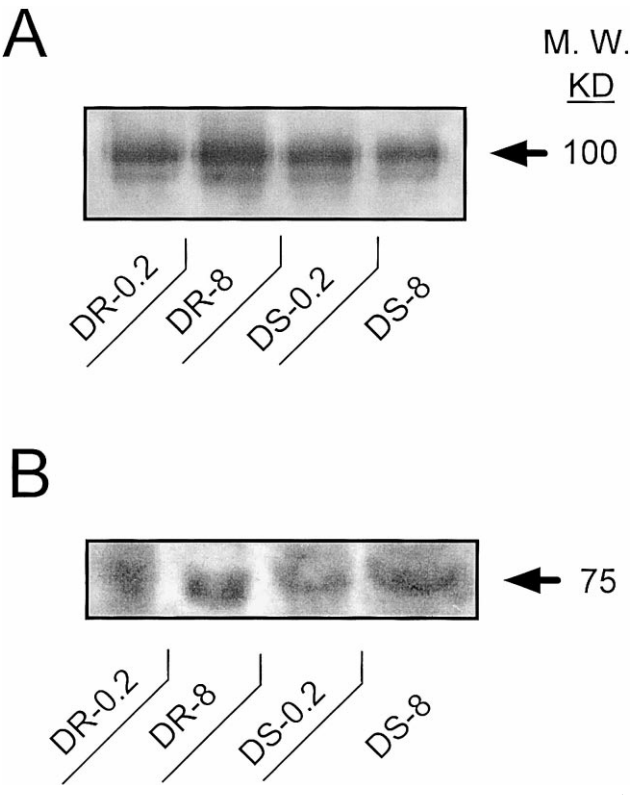

Fig. 1. Effects of high-salt load on the expression of Na^+, K^+-ATPase and SGLT1. (A) Renal BLMV fractions (20 μg) were prepared from normal-salt loaded DR rats (DR-0.2), high-salt loaded DR rats (DR-8), normal-salt loaded DS rats (DS-0.2), and high-salt loaded DS rats (DS-8). The arrow indicates the molecular mass of Na^+,K^+-ATPase. (B) Renal BBMV fractions were applied to gels and blotted with an anti-SGLT1 antibody. The arrow indicates the molecular mass of SGLT1.

amount of SGLT1 using BBMV (Fig. 1B). SGLT1 is a 75 kDa polypeptide. There are also no differences between high- and normal-salt groups, or between DR and DS rats.

3.2. Na^+,K^+-ATPase activity of BLMV

Ouabain (1 mM)-sensitive ATPase activity was referred to as Na^+,K^+-ATPase activity. Na^+,K^+-ATPase activity of DR rats was not affected by the high-salt load (Fig. 2A). To determine the apparent affinity (K_m) and maximum velocity (V_{max}) of Na^+, K^+-ATPase for ATP, the enzyme activities were measured in media containing various concentrations of ATP (0.125–10 mM) with fixed Na^+ and K^+ concentrations. The K_m values were identical for both normal-salt groups and the high-salt loaded DR rats (Table 1, 0.52–0.82 mM). These data were comparable with the values obtained from rat renal medulla (0.99 mM) [17] and kidney (0.68 mM) [18]. However, the K_m value of the high-salt loaded DS rat showed a 4-fold increase compared to the normal-salt loaded DS rat. There were no differences in the V_{max} values among all groups.

3.3. AMG uptake of BBMV

SGLT1 activity was assayed by measurement of AMG uptake using renal BBMV. To determine the K_m and V_{max} of SGLT1 for AMG, the activity of the SGLT1 was measured in media containing various concentrations of non-labeled AMG (0.125–10 mM) with fixed Na^+ and AMG concentrations. The K_m values were identical among normal-salt groups and high-salt loaded DR rats (Table 1, 0.20–0.23 mM). These data were comparable with

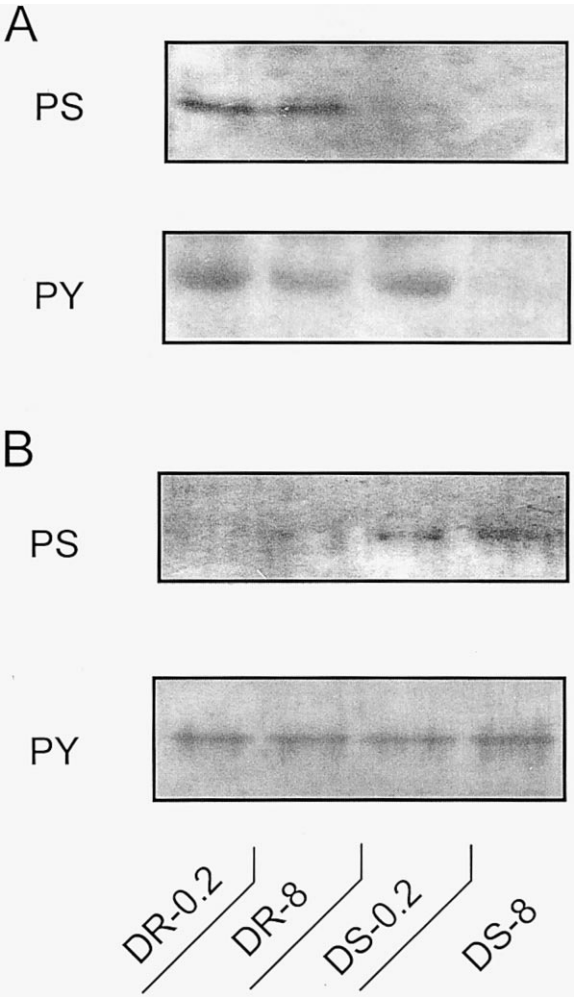

Fig. 2. Effects of the high-salt load on the amount of phosphorylated serine and tyrosine. (A) Membrane fraction of BLMV (40 μg) immunoprecipitated by an anti-Na^+,K^+-ATPase antibody were applied to the gel and blotted with an anti-phosphoserine (PS) or anti-phosphotyrosine antibody (PY). (B) Membrane fractions of BBMV (40 μg) immunoprecipitated by an anti-SGLT1 antibody were applied to the gel and blotted with an anti-phosphoserine (PS) or anti-phosphotyrosine antibody (PY).

Table 1
Summary of kinetic constants for Na^+,K^+-ATPase and SGLT1

	K_m		V_{max}	
	Na^+,K^+-ATPase (mM)	SGLT1 (mM)	Na^+,K^+-ATPase (μmol/mg/min)	SGLT1 (nmol/mg/min)
DR-0.2	0.52 ± 0.14	0.22 ± 0.03	0.47 ± 0.09	1.49 ± 0.12
DR-8	0.82 ± 0.34	0.20 ± 0.04	0.38 ± 0.12	1.55 ± 0.10
DS-0.2	0.56 ± 0.05	0.23 ± 0.03	0.42 ± 0.11	1.52 ± 0.06
DS-8	2.24 ± 0.38*	0.11 ± 0.01*	0.51 ± 0.15	2.56 ± 0.19*

Values are means ± S.E.M. from three independent measurements. The K_m and V_{max} values for ATP and AMG were estimated from Lineweaver–Burk plots. *$P < 0.05$, statistically significant differences among DR-0.2, DR-8, DS-0.2 and DS-8.

the values obtained from rat kidney (0.2 mM) [19] and rabbit intestine (0.36 mM) [9]. However, the K_m value of the high-salt loaded DS rat was 2-times lower than that of the normal-salt loaded DS rat. Furthermore, the V_{max} value of the DS rat was increased by about 1.7 times with the high-salt load.

3.4. Comparison of phosphorylated proteins

As described above, we found that the high-salt load induced a decrease in the affinity for Na^+,K^+-ATPase and an increase in the transport velocity of AMG in DS rats. We investigated whether the protein phosphorylation levels of Na^+,K^+-ATPase and SGLT1 were affected by the high-salt load. The Na^+,K^+-ATPase α subunit was phosphorylated by protein kinase C and A [2,8]. It has been reported that protein kinase A stimulation induced phosphorylation exclusively on Ser-943 and protein kinase C stimulation on Tyr-15 and Ser-16 [20,21]. Western blot analysis of immunoprecipitated BLMV samples was performed using an anti-phosphoserine or anti-phosphotyrosine antibody. The amount of phosphorylated serine and tyrosine was not affected by the high-salt load in DR rats. In contrast, the amount of phosphorylated tyrosine was decreased by the high-salt load in DS rats (Fig. 2A).

Next, we attempted to compare the amount of phosphorylated proteins in immunoprecipitated BBMV samples. SGLT1 is also phosphorylated by protein kinase C and A. The phosphorylation sites were deduced to be Tyr-50, Ser-303, Ser-418, and Tyr-635 [22]. When rat SGLT1 was phosphorylated by protein kinase A, the transport activity was increased [12]. On the contrary, protein kinase C mediated phosphorylation decreased the transport activity [10]. In the case of anti-phosphotyrosine, we cannot detect any difference between DR and DS rats, or between high- and normal-salt groups (Fig. 2B, lower). In the case of anti-phosphoserine, the amount of phosphorylated protein in the high-salt loaded DS rat was very high as compared to those of both the high-salt loaded DR rats and the normal-salt loaded DS rats (Fig. 2B, upper). This increase in phosphorylated protein was in agreement with the increase in V_{max}.

4. Discussion

The development of hypertension in DS rats is linked to genetic alterations of renal functions that determine the inability to Na^+ excretion [23]. These alterations precede the development of hypertension [24]. A single amino acid substitution in Na^+,K^+-ATPase altered the enzyme activity, and this substitution occurs in DS rats [25]. Nishi et al. [2] then reported that the kinetic parameters for K^+ activation were affected by a normal-salt load in DS rats, but for Na^+ activation was not affected. This difference of the K^+ affinity was found in the intact cells, but not in BLMV or the purified enzyme. In the present study, we used high-salt loaded DS rat, which already have hypertension. Abdelrahman et al. [26] reported that a high-salt load did not produce any significant change in the renal Na^+,K^+-ATPase activity in either DS or DR rats, and that there was no change in the composition of the α-subunit. We also showed that the amount of Na^+,K^+-ATPase protein was not changed by the high-salt load for 15 days. However, in contrast our results showed that the high-salt load potently inhibited Na^+,K^+-ATPase activity in DS rats. Recently, Cheng et al. [27] reported that effects of protein kinase A and C on Na^+,K^+-ATPase activity are dependent on the intracellular Ca^{2+} concentration. Therefore, we suggested that a difference in the procedure of vesicle preparation may have caused the diversity of results on Na^+,K^+-ATPase activity.

Na^+,K^+-ATPase activity is mediated by complex functional and structural intracellular networks, including the levels and distribution of protein kinase A and C (for a review see [28]). High-salt load may change the conformational transitions, phosphorylated conformations, and/or regulatory factors. Na^+,K^+-ATPase moves between the E1 and E2 forms to reverse transport of Na^+ and K^+ ions. The E1 form binds ATP and intracellular Na^+, and the E2 form binds extracellular K^+. If the high-salt load changed the conformational transitions, the affinity of the enzyme for ATP, Na^+, or K^+ may be altered. However, the kinetic parameters for Na^+ and K^+ were not changed in high-salt loaded DS rats [26]. We found that the affinity for ATP in

high-salt loaded DS rats was markedly decreased but with little change in the V_{max} value (Table 1). In DR rats, these parameters of K_m and V_{max} were not significantly affected by the high-salt load. The conformational transition was unchanged by the high-salt load, but a change in the substrate binding sites may occur in DS rats. The phosphorylations of Na^+, K^+-ATPase by protein kinase C and A affected the enzyme activity. In general, the activity of Na^+, K^+-ATPase phosphorylated by protein kinase C and/or protein kinase A are lowered than that of non-phosphorylated Na^+,K^+-ATPase [2]. We compared the phosphorylation level of Na^+,K^+-ATPase between high- and normal-salt loaded DS rats. The phosphorylated serine existed in DR rats, but it could not be detected in DS rats. Interestingly, the phosphorylated tyrosine was disappeared in high-salt loaded DS rats. (Fig. 2A). So far, it has been reported that the activities of protein kinase A and C are reduced in the spontaneously hypertensive rat [29,30]. At present we could not exclude the possibility that the phosphorylation levels of Na^+,K^+-ATPase were affected by some enzyme activity, which phosphorylated other amino acid residues other than serine and tyrosine.

Above, we described the high-salt load inhibition of Na^+,K^+-ATPase activity in BLMV from DS rat. We also examined the possibility that Na^+ transporters in the brush border membrane were affected by a high-salt load. SGLT1 is widely expressed in various tissues [31], and in kidney, SGLT1 has key roles in the reabsorption of glucose as well as that of Na^+. So far, the effect of a high-salt load on SGLT1 activity has not yet been reported. In the present study, we found that the SGLT1 activity in high-salt loaded DS rat was significantly increased compared with both normal-salt groups and the high-salt loaded DR rat. This increase was accompanied with an enhancement of the V_{max} value (Table 1). Protein kinase A activated SGLT1 in LLC-PK_1 cells [32], rabbit SGLT1 expressing *Xenopus laevis* oocytes [10] and rat small intestine [12]. On the contrary, protein kinase C inactivated SGLT1 in LLC-PK_1 cells [33], *X. laevis* oocytes [10] and COS-7 cells [34]. The putative phosphorylation site of protein kinase A has not been reported in rat SGLT1 [22]. The expression of SGLT1 protein in BBMV was not different between DR and DS rats, or between high- and normal-salt groups. The amount of phosphorylated serine in high-salt loaded DS rats was highest among the four groups, although the amount of phosphorylated tyrosine was no different from the others. Three serine residues are possibly phosphorylated in SGLT1 [35] and it is not yet know whether the tyrosine residue are phosphorylated in the intact cell. We suggest that protein kinase A directly phosphorylates SGLT1 and regulate its transport activity. Further investigation will be necessary to identify the sites which are phosphorylated by protein kinase A.

Na^+ is absorbed via SGLT1 on the brush border membrane and excluded via Na^+,K^+-ATPase on the basolateral membrane. In the present study, we found that the SGLT1 activity increased in high-salt loaded DS rats, although the Na^+,K^+-ATPase activity decreased. The kinetic parameters were also different: the high-salt load increased the K_m in Na^+,K^+-ATPase, while V_{max} was increased in SGLT1. These phenomena both lead to an increase in intracellular Na^+ concentration, and this increase in the intracellular Na^+ concentration may be a trigger for the development of hypertension.

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