Dysfunction of Paracellin-1 by Dephosphorylation in Dahl Salt-Sensitive Hypertensive Rats

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Abstract: A high-salt diet reduced the levels of renal cAMP content and serine-phosphorylated paracellin-1 in Dahl salt-sensitive hypertensive rats. In MDCK cells expressing paracellin-1, protein kinase A inhibitor reduced the serine-phosphorylated

paracellin-1 and transepithelial Mg^{2+} transport, suggesting that a dephosphorylation of paracellin-1 induces the reduction of Mg^{2+} reabsorption in salt-sensitive hypertension.

Key words: paracellin-1, phosphorylation, hypertension.

Arterial hypertension is the most common chronic disease in industrialized countries. Much evidence demonstrates a clear correlation between excess salt intake and the pathogenesis of hypertension [1–3]. The mechanism, however, by which excess salt intake elevates blood pressure is unknown. Dahl rats represent related strains that have an important difference in their response to a high-salt diet: the salt-sensitive (DS) strain develops hypertension, whereas the salt-resistant (DR) strain does not [4]. We [5] and others [6] reported that a high-salt diet in DS rats reduces the renal reabsorption of Mg^{2+} . Conversely, dietary magnesium supplementation attenuates the blood pressure in deoxycorticosterone acetate (DOCA) salt-hypertensive rats [7, 8]. These reports indicate that the body magnesium content is an important factor in salt-sensitive hypertension. In humans, several studies suggest that a decreased dietary intake of magnesium is associated with an increased risk of hypertension and that dietary magnesium supplementation lowers blood pressure [9–11].

Magnesium is an important cofactor for most of the ATPases. In the kidney, a magnesium deficiency eventually leads to low levels of calcium and potassium and to high levels of sodium and chloride [12]. This electrolytic imbalance may be a risk factor for hypertension. The body magnesium content is controlled by reabsorption through the renal epithelial cells. The renal Mg^{2+} filtrated in the glomeruli is predominantly reabsorbed by the paracellular

pathway in the thick ascending limb (TAL) of Henle [13]. Simon *et al.* [14] reported that paracellin-1 (named as claudin-16) is exclusively expressed in the cell–cell contact area, that is, the tight junction (TJ) of TAL, and it might play a critical role in the control of paracellular permeability for Mg^{2+} and Ca^{2+} . Indeed, we revealed that paracellin-1 expression increases in the paracellular transport of divalent cations [15]. Several claudins have been reported to construct size and charge selective pores [16–18]. However, the regulatory mechanisms of claudins expression are poorly understood.

In the present work, we show that a high-salt diet decreased the level of serine-phosphorylated paracellin-1 in DS hypertensive rats. In Madin-Darby canine kidney (MDCK) cells expressing FLAG-tagged paracellin-1, paracellin-1 was phosphorylated at serine residue under control conditions. Protein kinase A (PKA) inhibitor reduced the level of serine-phosphorylated paracellin-1, transepithelial electrical resistance (TER), and transepithelial Mg^{2+} transport. In contrast, protein kinase C (PKC) inhibitor reduced TER, but it did not affect transepithelial Mg^{2+} transport. A high-salt diet decreased the cAMP content in the renal cortex of DS rats. Our data suggest that a decrease in cAMP content induces the reduction of serine-phosphorylated paracellin-1 in DS hypertensive rats, resulting in a reduction of Mg^{2+} reabsorption.

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Abbreviations: MDCK, Madin-Darby canine kidney; DS, Dahl salt-sensitive; DR, Dahl salt-resistant; PKA, protein kinase A; PKC, protein kinase C; TER, transepithelial electrical resistance; H-89, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride.

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Materials and methods

Cell culture. Cell culture and transfection procedures for MDCK cells were described previously [15]. Stable transfectants of FLAG-tagged paracellin-1 were maintained in the continuous presence of the selecting drug (0.5 mg/ml G418).

Preparation of membrane fraction and immunoprecipitation. Inbred male DR and DS rats (Japan Clea, Tokyo, Japan) were divided into two groups, and each group had free access to a normal salt (0.2%) (DR-N and DS-N) or a high salt (8%) diet (DR-H and DS-H) for four weeks (each group contains four rats). The brush border membrane (BBM) fractions of the renal cortex from DR and DS rats were prepared with Mg^{2+} -EGTA precipitation methods [19]. The lysates of MDCK cells were prepared as described previously [15]. Protein concentrations were measured using the protein assay kit (Bio-Rad Laboratories, CA), with bovine serum albumin as the standard. In immunoprecipitation, the samples were solubilized in a lysis buffer containing 150 mM NaCl, 0.5 mM EDTA, 1% Triton X-100, and 50 mM Tris-HCl (pH 8.0) and were incubated with protein G-Sepharose beads and anti-paracellin-1 or anti-FLAG antibodies at 4°C for 120 min with gentle rocking. After centrifugation at $6,000 \times g$ for 1 min, the pellet was washed four times with a lysis buffer. The BBM fractions of the renal cortex, the lysate fractions of MDCK cells, and immunoprecipitates were solubilized in a sample buffer for SDS-PAGE.

Immunoblotting. Immunoblotting was performed with commercial antibodies raised against FLAG, phosphoserine (Sigma, MO) and paracellin-1 (Santa Cruz, CA). The specific binding of antibodies was detected with peroxidase-conjugated secondary antibodies and visualized by enhanced chemiluminescence detection (Amersham Biosciences, Buckinghamshire, UK).

Measurement of paracellular permeability. MDCK cells expressing FLAG-tagged paracellin-1 were plated at confluent densities on Transwells with polyester membrane inserts (Corning Life Sciences, MA). TER was measured using a Millicell-ERS epithelial volt-ohmmeter (Millipore, MA). TER values ($\text{ohms} \times \text{cm}^2$) were normalized by the area of the monolayer and calculated by subtracting the blank values from the filter and the bathing medium. The paracellular diffusion of FITC-dextran-4k from the apical to the basal compartments was measured with a Fluoroskan Ascent CF (Thermo Labosystems). The transepithelial transport of Mg^{2+} from the apical to the basal compartments was measured using xylydyl blue-I (XB-I). At time 0, a transport buffer (140 mM NaCl, 5.8 mM KCl, 0.34 mM Na_2HPO_4 , 0.44 mM KH_2PO_4 , 1 mM CaCl_2 , 25 mM glucose, and 20 mM Hepes, pH 7.4) supplemented with 10 mM Mg^{2+} was poured into the filter well, and the reverse compartment was filled with the transport buffer without Mg^{2+} . After incubation at 37°C for 60 min, the reverse compartment media were collected and subjected to

Fig. 1. Decrease of phosphorylated paracellin-1 by a high-salt diet. **(A)** The BBM fractions (50 μg) were immunoblotted with anti-paracellin-1 (IB: PCLN) or anti-actin antibodies (IB: actin). The BBM fractions (500 μg) were immunoprecipitated with protein G-Sepharose beads and anti-paracellin-1 antibody. The immune pellets were immunoblotted with anti-phosphoserine (IP: PCLN, IB: P-Ser) or anti-paracellin-1 (IP: PCLN, IB: PCLN) antibodies. **(B)** The band densities of immunoblotting were expressed relative to the values in DR-H, DS-N, and DS-H of the value in DR-N. $n = 3-4$. $**P < 0.01$.

Mg^{2+} measurement. XB-I formed a 520 nm absorbance maximum complex on Mg^{2+} binding under alkaline conditions.

Measurement of cAMP content and protein phosphatase-2A. The homogenized renal cortex from DR and DS rats was centrifuged at $10,000 \times g$ for 60 min, and supernatant was then collected. The supernatant was diluted appropriately and used for the assay. The cAMP content in renal cortex was assessed using the cAMP Assay Kit (Cayman Chemical Company, MI). The value of cAMP content was determined by absorbance at 405 nm. The

Fig. 2. Dysfunction of paracellin-1 by PKA inhibitor. (A) MDCK cells expressing FLAG-tagged paracellin-1 were treated with 50 μ M H-89 or 50 μ M calphostin C (Cal) for 60 min. The lysates of MDCK cells (30 μ g) were immunoblotted with anti-FLAG antibody (IB: FLAG). The lysates of MDCK cells (500 μ g) were immunoprecipitated with protein G-Sepharose beads and anti-FLAG antibody. The immune pellets were immunoblotted with anti-phosphoserine (IP: FLAG, IB: P-Ser) or anti-FLAG (IP: FLAG, IB: FLAG) antibodies. (B, C, D) TER values, FITC-dextran diffusion, and transepithelial Mg²⁺ transport from the apical to the basal compartments were measured in cells treated with 50 μ M H-89 or 50 μ M calphostin C (Cal) for 60 min. ** P < 0.01. NS, not significantly different from control.

protein phosphatase 2A (PP2A) activity in the renal cortex was assessed using the Serine/Threonine Phosphatase Assay System (Promega). The value of PPTase-2A activity was determined by absorbance at 630 nm.

Statistics. The results are presented as means $\pm$ SEM. The differences between groups were analyzed by a one-way analysis of variance, and correction for multiple comparisons was made using Tukey's multiple comparison test. Significant differences were assumed at P < 0.05.

Results and discussion

A high-salt diet contributes to the development of hypertension, but the cause of salt sensitivity is unknown. Several studies suggest that an inverse relationship exists between dietary Mg²⁺ intake and blood pressure changes. There is a possibility that an abnormal handling of Mg²⁺ is involved in the development of hypertension by a high-salt diet. We [5] and others [6] reported that a high-salt diet in DS rats reduces the renal reabsorption of Mg²⁺. However, the aberrant mechanisms are as yet unclear. Most Mg²⁺ is suggested to be reabsorbed by paracellin-1 in the TAL. At first we examined the expression and phosphorylation level of paracellin-1. The amount of actin protein was measured as a standard. A high-salt diet had no effect on the expression of paracellin-1 protein in both DR and DS rats (Fig. 1). Preliminarily, a reverse-transcriptase PCR showed that the amount of paracellin-1 mRNA is unaffected by a high-salt diet in both the DR and the DS rats (data not shown). Notably, a high-salt diet decreased the level of serine-phosphorylated paracellin-1 in DS rats,

whereas in DR rats it was invariant. These results suggest that the decrease in serine-phosphorylated paracellin-1 is involved in the abnormality of Mg²⁺ reabsorption in DS rats.

To clarify the significance of phosphorylation, we proceeded to the functional analysis using mammalian expression systems. Several claudins were reported to be phosphorylated by PKA [20, 21] or PKC [22]. Here we used the MDCK cells expressing stably FLAG-tagged paracellin-1. We recently reported that FLAG-tagged paracellin-1 is phosphorylated at serine residue under control conditions and a PKA inhibitor, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), decreases the phosphoserine level of paracellin-1 [23]. In contrast, a PKC inhibitor, calphostin C, slightly decreased the phosphoserine level of paracellin-1, but its effect was weaker than that of H-89 (Fig. 2A). The treatment of H-89 or calphostin C did not affect the amount of paracellin-1 protein expression. The effect of H-89 on the FLAG-tagged paracellin-1 in MDCK cells is similar to the decrease in serine-phosphorylated paracellin-1 in DS rats ingesting a high-salt diet, suggesting that a high-salt diet induced the decrease in serine-phosphorylated paracellin-1, mediated via dysfunction of the cAMP/PKA-dependent pathway. Dephosphorylated paracellin-1 is dissociated from the tight junction [23]. We suggest that an abnormality in the localization of paracellin-1 may cause the loss of its original function.

We next examined the effects of protein kinase inhibitors on the function of paracellin-1. H-89 and calphostin C

Fig. 3. Decrease of renal cAMP content by a high-salt diet. (**A, B**) The homogenate of renal cortex was prepared from DR-N, DR-H, DS-N, and DS-H rats. The cAMP content and the PP2A activity were measured as described in MATERIALS AND METHODS. * $P < 0.05$.

decreased TER without affecting the paracellular diffusion of FITC-dextran (Fig. 2, B and C). H-89 decreased transepithelial Mg^{2+} transport, but calphostin C did not (Fig. 2D). These reports support that paracellin-1 is phosphorylated by PKA, but not by PKC. Although H-89 completely inhibited the phosphorylation of paracellin-1, it decreased transepithelial Mg^{2+} transport by 25.8%. In untransfected MDCK cells, transepithelial Mg^{2+} transport was 83.8 ± 3.0 nmoles/well/h. This value was equal to that in the paracellin-1-expressing cells treated with H-89. We suggest that paracellin-1-independent Mg^{2+} transport pathways function in untransfected cells and the paracellin-1-expressing cells treated with H-89.

So far, it has been reported that forskolin, a cAMP agonist, phosphorylated claudin-3 without affecting the amount of claudin-3 protein and decreased in TER in ovarian cancer cells [20]. In contrast, CPT-cAMP, a cAMP analog, increased in the expression of claudin-5 and TER in endothelial cells [21]. Thus the response of claudins against cAMP is different in the types of claudins. In

MDCK and LLC-PK₁ cells, each exogenous claudin showed its own unique ion selectivity, and overexpression would overwhelm the endogenous claudins, thus making the final paracellular selectivity independent of the cell background [17]. We suggest that phosphorylated paracellin-1 made an Mg^{2+} permeable pore mediated by homophilic interactions paracellin-1 itself or heterophilic interactions between paracellin-1 and other claudins.

Protein phosphorylation and dephosphorylation are carried out by kinases and phosphatases, respectively. Umemura *et al.* [12] reported that urinary cAMP excretion and the renal adenylate cyclase response to parathyroid hormone are lower in DS than in DR rats at the ages of 3–32 weeks. This suggests that the renal cAMP content is lower in DS than in DR rats. Notably, we found that the cAMP content of renal cortex in DS rats ingesting a high-salt diet was lower than those in DS rats ingesting a normal-salt diet and in DR rats ingesting normal- and high-salt diets (Fig. 3A).

The reduction of serine-phosphorylated paracellin-1 might be caused not only by the decrease in cAMP content (PKA activity), but also by the increase in protein phosphatases, which are divided into two large groups: protein serine/threonine and tyrosine phosphatases [24, 25]. PP2A accounts for the major portion of serine/threonine phosphatase activity in most tissues and cells. Therefore we examined the PP2A activity in the renal cortex. In DS rats ingesting a high-salt diet, it was slightly higher than in other groups, but it was not significant (Fig. 3B). In spontaneously hypertensive rats, PP2A activity is not different from that in normotensive Wistar-Kyoto rats [26]. We suggest that PP2A activity does not closely relate to the regulation of renal cAMP content in hypertensive rats. At present, we cannot deny the involvement of other protein phosphatases such as PP1, PP2B, and PP2C because of the lack of selective inhibitors/activators for different protein phosphatases. The increase of Gi alpha-2 and Gi alpha-3 proteins preceded the development of blood pressure in DOCA-salt hypertensive rats [27], indicating that the decrease in the cAMP level is a factor that contributes to hypertension. In contrast, tubular Mg^{2+} reabsorption is upregulated by various hormones acting through cAMP production [28]. Therefore the renal cAMP content may be a key factor for the regulation of Mg^{2+} reabsorption and blood pressure.

In conclusion, we found that paracellin-1 is dephosphorylated in DS rats ingesting a high-salt diet. The phosphorylation of paracellin-1 is upregulated by PKA, but not by PKC. The functions of paracellin-1 such as an Mg^{2+} permeable pore appeared when paracellin-1 was phosphorylated by a cAMP/PKA-dependent pathway. The cAMP content of renal cortex in DS rats ingesting a high-salt diet was lower than in other groups. Although we cannot deny the involvement of other regulatory factors, the reduction of phosphorylated paracellin-1 may be caused by the im-

paired function of cAMP/PKA-dependent hormonal regulation in DS rats. The reduction of phosphorylated paracellin-1 may result in the decrease of magnesium reabsorption in DS rats.

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