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Phosphorylation of Na–Cl cotransporter by OSR1 and SPAK kinases regulates its ubiquitination

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ABSTRACT

Na–Cl cotransporter (NCC) is phosphorylated in its amino terminus based on salt intake under the regulation of the WNK-OSR1/SPAK kinase cascade. We have observed that total protein abundance of NCC and its apical membrane expression varies in the kidney based on the phosphorylation status. To clarify the mechanism, we examined NCC ubiquitination status in mice fed low, normal and high salt diets, as well as in a model mouse of pseudohypoaldosteronism type II (PHAII) where NCC phosphorylation is constitutively elevated. Low-salt diet decreased NCC ubiquitination, while high-salt diet increased NCC ubiquitination in the kidney, and this was inversely correlated with total and phosphorylated NCC abundance. In the PHAII model, the ubiquitination of NCC in kidney was also lower when compared to that in wild-type littermates. To evaluate the relationship between phosphorylation and ubiquitination of NCC, we expressed wild-type, phospho-deficient and -mimicking NCC in COS7 cells, and the ubiquitination of immunoprecipitated total and biotinylated surface NCC was evaluated. NCC ubiquitination was increased in the phospho-deficient NCC and decreased in phospho-mimicking NCC in both total and surface NCC. Thus, we demonstrated that NCC phosphorylation decreased NCC ubiquitination, which may contribute to the increase of NCC abundance mostly on plasma membranes.

1. Introduction

The thiazide-sensitive NaCl cotransporter (NCC) is essential for sodium reabsorption at the distal convoluted tubules (DCT) in the kidney. A loss-of-function NCC mutation causes Gitelman's syndrome, an inherited disease that exhibits salt-losing phenotypes [1]. In contrast, a gain-of-function mutation in NCC causes pseudohypoaldosteronism type II (PHAII), a disease of salt-sensitive hypertension. PHAII was shown to be caused by mutations in the with-no-lysine kinases 1 and 4 (WNK1 and WNK4) [2], and we clarified using a PHAII model mouse (*Wnk4*$^{D561A/+}$) that the pathogenesis of PHAII was the constitutive activation of a phosphorylation signal cascade consisting of WNK kinase, oxidative stress-responsive kinase-1 (OSR1), STE20/SPS1-related proline/alanine-rich kinase (SPAK), and NCC [3].

NCC is phosphorylated at several amino-terminal serine and threonine residues (T53, T58 and S71 in mouse NCC) by OSR1/SPAK [4], which is highly elevated in *Wnk4*$^{D561A/+}$ knock-in mice [3]. SPAK knockout mice showed decreased phosphorylation of NCC at these sites and exhibit Gitelman's syndrome-like phenotypes [5], thus confirming the importance of NCC phosphorylation in the *in vivo* function of NCC. Pacheco-Arevarez et al. showed that the phosphorylation of NCC is important for its transport function when expressed in *Xenopus* oocytes [6]. In addition to this mechanism, it was observed that phosphorylated NCC was concentrated on the plasma membranes of DCT, thus suggesting that phosphorylation is involved in the accumulation of NCC in plasma membranes [7,8].

We recently reported several physiological regulators of NCC phosphorylation. NCC phosphorylation is increased by a low-salt diet through aldosterone [9]. Angiotensin II and insulin were found to increase NCC phosphorylation [10–13]. Interestingly, total protein abundance of NCC appeared to vary according to NCC phosphorylation in these cases. Similar phenomena were also observed in *Wnk4*$^{D561A/+}$ knock-in, SPAK knockout, SPAK and OSR1 knock-in mice [3,5,14], thus suggesting that NCC phosphorylation is able to regulate its total protein abundance mostly on plasma membranes. However, the underlying mechanisms of how phosphorylation of NCC increases surface expression have yet to be clarified.

Numerous membranous proteins, including various ion transporters of the kidney, have been shown to be degraded in a regulated manner that involves ubiquitination. For example, epithelial sodium channel (ENaC) is ubiquitinated by Nedd4-2, and ubiquitinated ENaC is sorted to a degradation pathway [15]. Impaired ENaC ubiquitination at the cell surface by the mutation of ENaC in Liddle

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syndrome results in increased ENaC protein levels at the cell surface, leading to increased sodium reabsorption and hypertension [16,17]. In the case of NCC, it has been reported that NCC is polyubiquitinated in its secretory pathway (ER) and undergoes proteasomal degradation [18]. It has also been reported that ubiquitin ligase Nedd4-2 ubiquitinates NCC [19]. These observations suggest that NCC phosphorylation and ubiquitination are coordinated and involved in the regulation of NCC under various pathophysiological conditions.

In this study, we found that dietary salt intake regulates NCC ubiquitination and phosphorylation *in vivo*. Similarly, NCC ubiquitination was decreased in a PHAII mouse model. In addition, we clarified that NCC phosphorylation regulates NCC ubiquitination. Thus, ubiquitination of NCC may be an important determinant of NCC protein abundance and plasma membrane localization within cells.

2. Materials and methods

2.1. Animal study

C57BL/6 mice (age, 12 weeks) were fed normal diet, low-NaCl diet (0.01% NaCl (w/w)) or high-NaCl diet (4% NaCl (w/w)) for 14 days. *Wnk4*$^{+/+}$ and *Wnk4*$^{D561A/+}$ mice were fed normal diet. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Tokyo Medical and Dental University.

2.2. Immunoprecipitation of NCC from mouse kidney

Mice were sacrificed after 14 days on the diet. Whole kidneys were homogenized in homogenization buffer (250 mM sucrose, 10 mM triethanolamine, 1 mM EGTA, 1 mM EDTA, 20 mM *N*-ethylmaleimide (NEM), 50 mM sodium fluoride, 1 mM sodium orthovanadate and complete protease inhibitor). Isolated crude membrane fraction (17,000*g*) was solubilized in buffer SB (0.5% sodium-deoxycholate, 20 mM Tris HCl, 5 mM EDTA, 10% glycerol and complete protease inhibitor). Samples were precleared by incubating with immobilized protein G, and were then subjected to NCC immunoprecipitation by rabbit anti-NCC antibody (Chemicon, Temecula, CA, USA). As a negative control, samples were incubated with rabbit IgG.

2.3. Plasmids

Full-length wild-type T7-tagged NCC and T7-tagged phospho-deficient NCC expression plasmid (pRK5-T7-tagged NCC and pRK5-T7-tagged NCC) was kindly provided by Dr. T. Moriguchi [20]. T7-tagged phospho-mimicking NCC (pRK5-T7-tagged NCC) was generated by site-directed mutagenesis with using the QuikChange Mutagenesis system (Stratagene, La Jolla, CA, USA).

2.4. Cell culture and transient transfection

COS7 cells were grown in low bicarbonate Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum. Cells were transfected with 8 μg of each plasmid using Lipofectamine 2000 (Invitrogen, CA, USA).

2.5. Immunoprecipitation of transiently expressed NCC in COS7 cell

Empty vector (PRK-5), T7-tagged phospho-deficient, wild-type or phospho-mimicking NCC was transfected into COS7 cells. At 48 h after transfection, cells were lysed with buffer M-PER (Thermo Scientific, Massachusetts, USA) containing 20 mM NEM and complete protease inhibitor. Lysates were centrifuged at 17,000*g* for 30 min. Supernatant was precleared with immobilized protein G, and was then subjected to NCC immunoprecipitation by rabbit anti-NCC antibody bound to protein A-Sepharose beads.

2.6. Cell surface biotinylation assay

COS7 cells were transfected with empty vector (PRK-5), T7-tagged phospho-deficient, wild-type or phospho-mimicking NCC. Cell surface proteins were labeled with sulfo-NHS (*N*-hydroxysuccinimido)-SS-biotin (Thermo Scientific) at 48 h after transfection as follows: cells were washed three times with PBS-CM (PBS with 1 mM $MgCl_2$ and $CaCl_2$). Cells were incubated for 30 min on ice with 0.5 mg/ml sulfo-NHS-SS-biotin in PBS-CM, and were then quenched with 100 mM glycine in PBS-CM. After washing three times with PBS-CM, cells were lysed in buffer M-PER (Thermo Scientific) containing 20 mM NEM and complete protease inhibitor. Lysates were centrifuged at 17,000*g* for 30 min. For pull down of total surface protein, lysates were incubated with immobilized NeutrAvidin beads (Pierce) at 4 °C for 2 h. For pull down of cell surface NCC, lysates were precleared with immobilized protein G, and were then subjected to the NCC immunoprecipitation by rabbit anti-NCC antibody (Chemicon, Temecula, CA, USA). NCC immune complexes bound to the protein A-Sepharose beads were eluted by boiling for 15 min in 100 μl of 1% SDS in PBS (pH 7.2) and diluted with 900 μl of PBS (pH 7.2). The solution was then incubated for 1 h at 4 °C with immobilized NeutrAvidin beads (Thermo Scientific) to isolate the biotinylated NCC surface fraction.

2.7. Immunoblotting

Semi-quantitative immunoblotting was performed, as described previously [21,22]. To assess relative expression levels of proteins in whole kidney, homogenates without the nuclear fraction (600*g*) or the crude membrane fraction (17,000*g*) were used. Band intensity was analyzed using Image J (NIH, Betheda, MD, USA). To quantify ubiquitinated protein signals from immunoblots, we calculated the amount of protein (in arbitrary units) from a 6-fold dilution series of the same protein that was also immunoblotted. The following primary antibodies were used in this study: rabbit total anti-NCC [23]; guinea pig total anti-NCC [23]; rabbit anti-pNCC (T53, T58 and S71) [9]; anti-T7 (from Invitrogen); and mouse anti-ubiquitin (Santa Cruz Biotechnology, CA, USA). Alkaline-phosphatase-conjugated anti-IgG antibodies (Promega, Madison, WI, USA) were used as secondary antibodies for immunoblotting.

2.8. Statistical analysis

Statistical significance was evaluated by unpaired *t*-test. All data are expressed as mean ± SEM. When more than three groups were compared, one-way ANOVA with Fischer's post hoc test was used. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Dietary salt regulates ubiquitination of NCC in mouse kidney

In order to investigate whether ubiquitination of NCC is involved in the regulation of its abundance in the kidney, we examined NCC ubiquitination in the kidney from mice under various dietary salt intake conditions. As shown in Fig. 1A, NCC protein abundance and phosphorylation were increased under a low-salt diet and decreased under a high-salt diet, as reported previously [9]. NCC ubiquitination was then evaluated under the same conditions. As shown in Fig. 1A and B, ubiquitination of NCC was significantly elevated and reduced in kidneys from mice fed high-salt and low-salt diets, respectively.