

# Dietary salt modulates the sodium chloride cotransporter expression likely through an aldosterone-mediated WNK4-ERK1/2 signaling pathway

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**Abstract** WNK is a serine/threonine kinase. Mutation in WNK1 or WNK4 kinase results in pseudohypoaldosteronism type II (PHA II) featuring hypertension, hyperkalemia and metabolic acidosis. Sodium chloride cotransporter (NCC) is known to be regulated by phosphorylation and trafficking. Dietary salt and hormonal stimulation, such as aldosterone, also affect the regulation of NCC. We have previously reported that WNK4 inhibits NCC protein expression. To determine whether dietary salt affects NCC abundance through WNK4-mediated mechanism, we investigated the effects of dietary salt change with or without aldosterone infusion (1 mg/kg/day) on NCC and WNK4 expression in rats. We found that high-salt (HS, 4% NaCl) diet significantly inhibits NCC mRNA expression and protein abundance while enhancing WNK4 mRNA and protein expression, whereas low-salt (LS, 0.07% NaCl) diet increases NCC mRNA

expression and protein abundance while reducing WNK4 expression. We also found that aldosterone infusion in HS-fed rats increases NCC mRNA expression and protein abundance, but decreases WNK4 expression. Administration with spironolactone (0.1 g/kg/day) in LS-fed rats decreases NCC mRNA expression and protein abundance while increasing WNK4 expression. We further showed that ERK1/2 phosphorylation was increased in HS-fed rats, but decreased in LS-fed rats. In HEK293 cells, over-expressed WNK4 increases ERK1/2 phosphorylation, whereas knockdown of WNK4 expression decreases ERK1/2 phosphorylation. Aldosterone treatment for 3 h decreases ERK1/2 phosphorylation. These data suggest that dietary salt change affects NCC protein abundance in an aldosterone-dependent mechanism likely via the WNK4-ERK1/2-mediated pathway.

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## Introduction

WNK (with no lysine (k)) is a serine/threonine kinase. Mutation in two members of this kinase, WNK1 and WNK4, causes pseudohypoaldosteronism type II (PHA II) featuring hypertension, hyperkalemia and metabolic acidosis [25]. The emerged evidence indicates that WNK kinase constitutes a novel signaling pathway regulating the ion channels and transporters maintaining electrolyte homeostasis [4]. Sodium chloride cotransporter (NCC) has been shown to be regulated by both phosphorylation and trafficking [11]. Dietary salt and hormonal stimulation, such as aldosterone, is also involved in the regulation

of NCC [19, 30]. We have previously shown that WNK4 wild type (WT) inhibits NCC protein expression, whereas PHA II-causing mutant or dead kinase mutant, D321A, loses its inhibitory effect on NCC [1]. We and other investigators also showed that WNK4 inhibits NCC by enhancing NCC degradation through a lysosomal pathway and/or interfering the forward trafficking of NCC [1, 3, 21, 32], indicating that WNK4 inhibits NCC activity by reducing NCC protein abundance. WNK1 and WNK4 have been shown to modulate NCC activity through STE20-related kinases, SPAK/OSR1 [14, 15]. A recent study reveals that angiotensin II stimulates NCC activity through WNK4 and SPAK kinases [18], indicating that WNK4–SPAK signaling stimulates NCC activity. In addition, aldosterone has been implicated in activation of NCC through an SGK1 and WNK4 signaling pathway *in vitro* [17]. Another study reported that activation of PKC by a phorbol ester was found to downregulate NCC function via activation of extracellular signal-regulated kinase (ERK) 1/2 kinase [7]. The kinase domain of WNK has a similarity to the mitogen-activated protein (MAP)/extracellular signal-regulated protein kinase (ERK) kinase (MEK) family [27]. Lately, it has been reported that WNK1 activates ERK5 by an MEKK2/3-dependent mechanism [28], and PKB mediates the phosphorylation of WNK1 at Thr-60 *in vivo* [22]. WNK2 was found to inhibit cell proliferation by negatively modulating the activation of MEK1/ERK1/2 [12]. WNK4 was found to increase ERK1/2 phosphorylation in response to hypertonicity and EGF stimulation [20]. However, what the WNK4-mediated downstream signaling is involving NCC regulation and whether WNK4 inhibits NCC activity and protein expression through an alternative signaling pathway, especially in *in vivo*, remain to be further explored. To this end, we reported that high-salt (HS) diet inhibits NCC mRNA expression and protein abundance while enhancing WNK4 mRNA and protein expression, whereas low-salt (LS) diet increases NCC mRNA expression and protein abundance while reducing WNK4 expression in rat. We also found that aldosterone infusion in HS-fed rats increases NCC mRNA expression and protein abundance while decreasing WNK4 expression. Administration with spironolactone in LS-fed rats decreases NCC mRNA expression and protein abundance while increasing WNK4 expression. We further showed that ERK1/2 phosphorylation is increased in HS-fed rats, but decreased in LS-fed rats. In HEK293 cells, over-expressed WNK4 increases ERK1/2 phosphorylation, whereas knockdown of WNK4 expression decreases ERK1/2 phosphorylation. Aldosterone treatment for 3 h decreases ERK1/2 phosphorylation. These data suggest that dietary salt change affects NCC protein abundance in an aldosterone-dependent mechanism likely through the WNK4-ERK1/2-mediated pathway.

## Materials and methods

### Animal studies

All animal experiments were approved by the Institutional Animal Care and Use Committee at Hua Shan Hospital, Fudan University School of Medicine, and Emory University School of Medicine. The experiments were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. All experiments were performed on male Sprague–Dawley rats (200–250-g body weight) that were kept under diurnal light conditions and had free access to food and water (Shanghai Laboratory Animal Center). All rats were fed a pelleted diet with 0.4% NaCl (NS) for 3 days, then randomly switched to five groups: continuously with 0.4% NaCl, a pelleted 4% NaCl (HS) diet, a gelled 0.07% NaCl (LS) diet, HS diet plus aldosterone (H+A) (Sigma) by osmotic mini-pump at 1 mg/kg/day [6, 9] and LS diet plus spironolactone (L+S) (Sigma) by intragastric administration at 0.1 g/kg/day all for 2 weeks. All diets were purchased from Shanghai Laboratory Animal Center. None of the rats showed significant loss of body weight during the experiment. At the beginning and end of the experiments, mean blood pressure was measured using the tail-cuff method, and 24-h urine was collected in the metabolic cage for determination of the rates of excretion of sodium and potassium (Hitachi 7170S automatic analyzer). After 2 weeks of treatment, the rats were sacrificed for semiquantitative immunoblotting and RT-PCR as described in the following. Rats were anesthetized using isoflurane for procedures and at sacrifice. Serum samples were collected at the time each rat was killed for determination of the renal function, plasma sodium and potassium and serum aldosterone concentration by radioimmunoassay.

### Cell culture and transfection

HEK293 cells were maintained in DMEM/F12 (1:1) (Invitrogen, CA) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and 10 % fetal bovine serum. Lipofectamine 2000 (Invitrogen) was used for transfection of plasmids into HEK293 cells according to the manufacturer's instructions. Opti-MEM medium was obtained from Invitrogen. Forty-eight hours after transfection, cell lysates were used for western blot. Lipofectamine RNAiMax (Invitrogen, CA) was used for transfection of WNK4 siRNA into HEK293 cells. The sequences of siRNAs used for the WNK4 knockdown include sense 5' CGG GCA CGC UCA AGA CGU AUU and antisense 5' P UAC GUC UUG AGC GUG CCC GUU [8]. The synthetic siRNAs were obtained from Invitrogen. For transfection, 5-µl 20-mM

scramble siRNA or WNK4 siRNA duplexes were added into 0.65-ml Opti-MEM and mixed gently. Ten microliters of Lipofectamine RNAiMAX was added into the diluted siRNA solutions and mixed gently, incubated at room temperature for 15~20 min to allow siRNAs-liposome complex to form. The Opti-MEM solution containing siRNA liposome complex was added to a 3.35-ml HEK293 cell suspension, and the mixture was plated in 60-mm dishes. Twenty-four hours after transfection, cells reached 60~70% confluences, and additional plasmid was transfected into the HEK293 cells if needed.

#### Western blot analysis

Western blot analysis was performed as previously described to determine protein levels of NCC, WNK4 and phosphorylated ERK1/2 using the homogenates from the total kidneys of the rats prepared as described [19]. The protein homogenate was not centrifuged to remove crude membrane fraction. The homogenates were prepared in lysis buffer containing phosphatase inhibitors. The protein sample was separated by SDS-polyacrylamide gel electrophoresis. After transferring, the membrane was probed with specific antibodies and detected using ECL. The antibodies for NCC, WNK4 and phosphorylated ERK1/2 were rabbit polyclonal or monoclonal antibodies purchased from Millipore (1:1,500), Abcam (1:100) and Cell Signaling (1:500), respectively. The molecular weights of NCC, WNK4 and phosphorylated ERK1/2 are indicated at 135, 160 and 42–44 kDa, respectively.

#### Real-time RT-PCR

Total kidney RNA was extracted using Trizol (Invitrogen, Carlsbad, California) and treated with DNase (Takara Bio Inc, Otsu, Shiga, Japan) according to the manufacturer's protocol. RNA was reverse-transcribed using the reverse transcription system kit (Promega, Madison, WI) as described in the manufacturer's protocol. PCR was performed with 2- $\mu$ l cDNA in a final volume of 50  $\mu$ l containing 1 $\times$  Ex Taq HS buffer (Takara Bio Inc, Otsu, Shiga, Japan), 10  $\mu$ M of sense primer and antisense primer, 1-mM dAGCU,

250-mM UNG, 10- $\mu$ M probe and 5 U/ $\mu$ l Ex Taq HS (Takara Bio Inc, Otsu, Shiga, Japan). To normalize the samples for absolute RNA amount, a  $\beta$ -actin-PCR was performed. Real-time PCR was carried out in an iCycler (Bio-Rad, Hercules, CA) using the following thermal cycling profile: 37°C for 5 min, 95°C for 3 min, followed by 40 cycles of amplification (94°C for 20 s, 60°C for 20 s). All samples were run in triplicate (Table 1).

#### Statistical analysis

The data are presented as the means  $\pm$  SE. Statistical significance was determined using either a Student's *t*-test when two groups were compared or by a one-way ANOVA, followed by Bonferroni's post-hoc tests, when multiple groups were compared. We assigned significance at  $p < 0.05$ .

## Results

#### Effects of dietary salts with or without aldosterone on blood pressure, plasma and urinary electrolytes

After 2 weeks of modified dietary salt feeding in the presence or absence of aldosterone infusion with or without spironolactone treatment, the plasma and urinary sodium and potassium levels, as well as plasma aldosterone level, were determined in different rats group. As shown in Table 2, at the end of dietary salt modification, there is no significant difference in total body weights among these dietary salt treatment groups. The systolic artery pressure (SAP) using direct measurement via the carotid artery was significantly increased from baseline to  $151 \pm 5$  vs.  $110 \pm 2$  mmHg ( $p < 0.05$ ,  $n = 8$ ) in the high salt with aldosterone infusion (H+A) group, while there was no significant change in SAP among other rat groups. In the high-salt (HS)-fed rat group, urinary sodium excretion was significantly increased ( $2,173 \pm 1241$  mmol/24 h,  $p < 0.05$ ,  $n = 8$ ) from baseline ( $752 \pm 180$  mmol/24 h); in the low-salt (LS)-fed rat group, urinary sodium excretion

**Table 1** Sense primer, antisense primer and probe for NCC, WNK4 and  $\beta$ -actin

	Sense primer	Antisense primer	Probe
NCC	5'-TCT ACT GGC TGT TTG ACG ATG G-3'	5'-CCT TTC TCT CTT CAT CCA TCC TGT-3'	5'-(FAM)-CTC ATC CCT TAT CTC CTG CAC CGC A-3'(TAMRA)
WNK4	5'-AGT GTC TCA GAC CAG AGC GAC AGA-3'	5'-AGA TCA AAT CGG AAC GTC ACC AT-3'	5'-(FAM)-CGT GGA GTG CCA GCT GCA AAC TCA C-3'(TAMRA)
$\beta$ -actin	5'-AAG ATG ACC CAG ATC ATG TTT GAG-3'	5'-TAG ATT GGC ACA GTG TGG GTG-3'	5'-(FAM)-ACC TTC AAC ACC CCA GCC ATG TAC GT-3'(TAMRA)

**Table 2** Body weight, blood pressure and urinary parameter from the different salt diets- fed rat groups

Group		H + A	HS	NS	LS	L + S
Final body weight (g)		323±23	317±17	315±16	312±21	310±20
SAP (mmHg)	Baseline	110±2	121±6	115±6	118±8	113±2
	After	151±5*	122±4	116±5	110±7	107±1
Urine Vvolume /24 h (ml)	Baseline	7.1±3.5	9.3±4.5	7.8±2.6	8.6±2	18.6±1.8
	After	10.5±2.4	14.4±3.3*	7.7±2.4	9±2.7	21.2±3.5
Urine Na/24 h (mmol)	Baseline	911±294	752±180	884±182	1,087±120	870±185
	After	954±34	2,173±1,241*	870±156	249±74*	1,480±210*
Urine K/24 h (mmol)	Baseline	1,135±156	1,113±61	1,246±120	1,303±130	1,353±185
	After	1,676±159*	887±81*	1,223±106	1,567±155*	1,793±138*

Rats were divided into five groups fed with different salt diets for 2 weeks. In addition, rats were treated with either aldosterone infusion or spironolactone. All of the data were collected using metabolic cages before the beginning of the experiments (baseline) and after completing the experiments (after)

LS low-salt diet (0.07%), NS normal-salt diet (0.4 %), HS high-salt diet (4%)

\* $p < 0.05$  in the post-treatment group compared to its respective baseline,  $n = 8$

was significantly reduced ( $249 \pm 74$  mmol/24 h,  $p < 0.05$ ,  $n = 8$ ) from baseline ( $1,087 \pm 120$  mmol/24 h). Urinary potassium excretions were significantly increased in the H+A and LS-fed groups compared to that in the normal-salt (NS)-fed group, indicating an effect of high level of aldosterones, whereas urinary potassium excretion was significantly reduced in the HS-fed group compared to that in the NS-fed group. The plasma sodium and potassium levels did not significantly change from baseline even though there was a trend of decrease in plasma potassium level in the H+A group (Table 3). As shown in Table 3, the plasma aldosterone level was decreased in the HS-fed rat group compared to that in the NS-fed rat group. In the LS-fed rat group, aldosterone level was increased. In the H+A group, the plasma aldosterone level was increased compared to the HS-fed group. There is no significant difference in plasma creatinine and chloride levels among these dietary salt treated groups (Table 3).

#### Effects of changes in dietary salts and aldosterone on NCC and WNK4 mRNA expressions

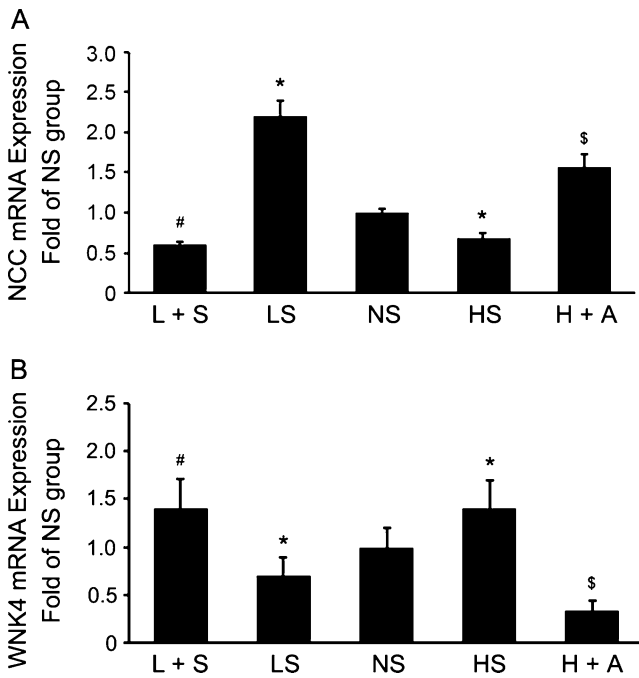
After 2 weeks of dietary modification, we investigated the effects of dietary salt changes on mRNA expression of sodium chloride cotransporter (NCC) in the kidneys using real-time PCR. In the HS-fed rat group, the NCC expression was decreased, whereas in the LS-fed rat group, NCC expression was increased, as seen in Fig. 1a. The NCC expression was increased in the H+A rat group compared with that of the HS-fed rat group, whereas the NCC expression was decreased in the LS with spironolactone (L+S) rat group compared with that of the LS-fed rat group. On the contrary to NCC mRNA expression levels, as seen in Fig. 1b, WNK4 mRNA expression was markedly enhanced in the HS-fed rat group and decreased in the LS group. But, it was decreased in the H+A rat group and enhanced in the L+S rat group. These data suggest that dietary salt changes affect NCC mRNA and WNK4 mRNA expressions in an inverse manner.

**Table 3** Plasma aldosterone, creatinine and electrolytes levels from the different salt diets- fed rat groups

Group	H + A	HS	NS	LS	L + S
Plasma K (mmol/L)	4.7±0.3	5.9±0.41	5.3±0.31	5.2±0.44	5.7±0.2
Plasma Na (mmol/L)	141.7±0.4	141.1±3.2	140.3±1.6	142.3±2.2	143.2±1.6
Plasma Cl (mmol/l)	102±0.1	101±1.8	101±1.5	103±1.2	102±1
Creatinine (μmol/L)	26.2±4.6	27.2±2.17	27.2±3.1	26.1±3.4	25.8±1.9
Aldosterone (μg/L)	2.12±0.18* <sup>#</sup>	0.50±0.05*	1.01±0.17	1.60±0.18*	1.83±0.25*

Rats were divided into five groups fed with different salt diets for 2 weeks with or without either aldosterone infusion or spironolactone. Plasma aldosterone, creatinine and electrolyte level were measured at the end of 2 weeks of dietary salt modifications

\* $p < 0.05$  compared to the NS group; <sup>#</sup> $p < 0.05$  compared to the HS group,  $n = 8$



**Fig. 1** NCC and WNK4 mRNA expressions in SD rats fed with different salt diets and treatments. NCC and WNK4 mRNA expressions were determined by real-time PCR in various SD rat groups. The mRNA expression is presented as the fold change from those in the NS-fed rat group. *NS* normal-salt diet, *LS* low-salt diet, *HS* high-salt diet, *L+S* LS group treated with spironolactone, *H+A* HS group treated with aldosterone. Note: # $p < 0.05$  compared to the LS group; \* $p < 0.05$  compared to the NS group; \$ $p < 0.05$  compared to the HS group

#### Effects of changes in dietary salts and aldosterone on NCC and WNK4 protein expressions

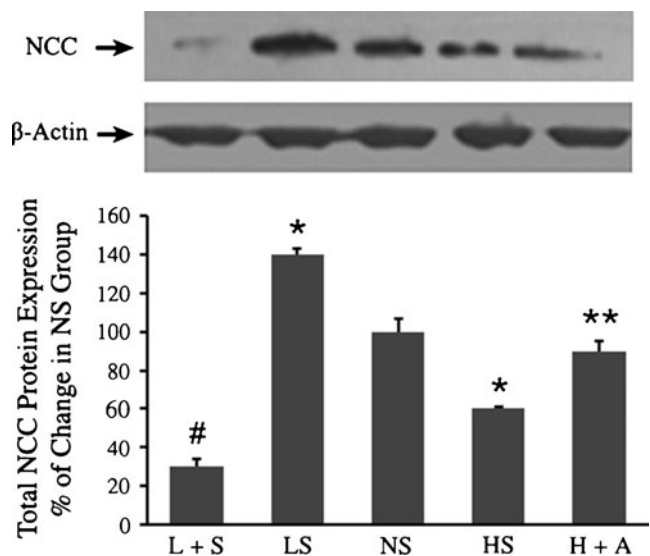
We further determined whether dietary salt changes affect NCC and WNK4 protein expression in a way similar to mRNA expression. As shown in Figs. 2 and 3, NCC protein abundance in the LS-fed rat group was significantly increased compared to that in the NS-fed rat group ( $p < 0.05$ ,  $n = 8$ ), while WNK4 protein expression in the same LS-fed rat group was significantly reduced. In the LS-fed rat group treated with spironolactone, NCC level was significantly reduced, while WNK4 protein level was significantly increased. NCC protein level in the HS-fed rat group was significantly decreased compared to the NS-fed rat group ( $p < 0.05$ ,  $n = 8$ ), while WNK4 protein level was significantly increased. When HS-fed rats were simultaneously administrated with aldosterone, NCC and WNK4 protein levels were significantly increased and decreased, respectively, compared to the HS-fed rat group ( $p < 0.05$ ,  $n = 8$ ). These findings suggest that dietary salts and aldosterone modulate NCC and WNK4 protein abundances in a similar way as their mRNA expressions.

#### Effects of change in dietary salt and aldosterone on ERK1/2 phosphorylations

Previous studies have shown that WNK4 phosphorylates ERK1/2 kinase [20], and the activated ERK1/2 phosphorylation by phorbol ester reduced NCC function in cell models [7]. Therefore, we further investigated whether the change of dietary salts affects ERK1/2 phosphorylation in the kidney. As shown in Fig. 4, in the HS-fed rat group, ERK1/2 phosphorylation was significantly increased compared to that in the NS-fed rat group, whereas in the LS-fed rat group, ERK1/2 phosphorylation was decreased compared to that in the NS-fed rat group. In the HS-fed rat group with aldosterone infusion, however, ERK1/2 phosphorylation was significantly decreased compared with that in the HS-fed rat group. When LS-fed rat was simultaneously administrated with spironolactone, a mineralocorticoid receptor antagonist, ERK1/2 phosphorylation decreased by aldosterone was significantly reversed compared to that in the LS-fed rat group. These data suggest that dietary salts affect NCC protein expression likely through an aldosterone-mediated WNK4-ERK1/2 signaling pathway.

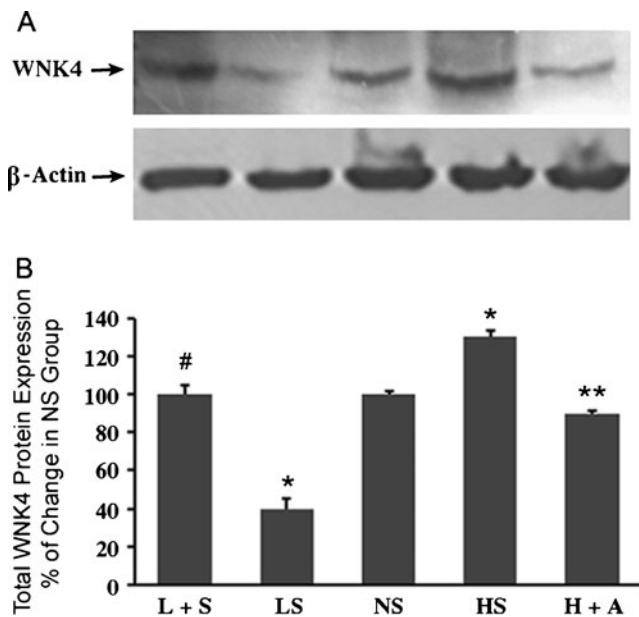
#### Effects of the WNK4 over-expression and knockdown of WNK4 expression on ERK1/2 phosphorylation in HEK293 cells

To confirm whether WNK4 modulates the MAPK ERK1/2 signaling pathway, we transiently transfected HEK293 cells



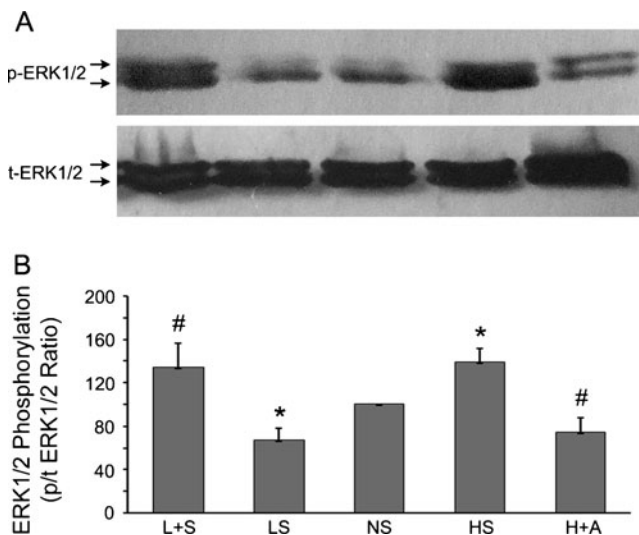
**Fig. 2** NCC protein expression in SD rats fed with different salt diets and treatments. NCC protein expression in different groups is presented as the percentage change of NCC in the NS group. *NS* normal-salt diet, *LS* low-salt diet, *HS* high-salt diet, *L+S* LS group treated with spironolactone, *H+A* HS group treated with aldosterone. Note: # $p < 0.05$  compared to the LS group; \* $p < 0.05$  compared to the NS group; \*\* $p < 0.05$  compared to the HS group





**Fig. 3** WNK4 protein expression in SD rats fed with different salt diets and treatments. WNK4 protein expression in different groups is presented as the percentage change of WNK4 in the NS group. *NS* normal-salt diet, *LS* low-salt diet, *HS* high-salt diet, *L+S* LS group treated with spironolactone, *H+A* HS group treated with aldosterone. Note: # $p < 0.05$  compared to the LS group; \* $p < 0.05$  compared to the NS group; \*\* $p < 0.05$  compared to the HS group

with different doses of WNK4 WT and performed western blot analysis. As shown in Fig. 5a and c, the over-expression of WNK4 significantly enhanced ERK1/2 phosphorylation in a dose-dependent manner. We further



**Fig. 4** ERK1/2 phosphorylation in SD rats fed with different salt diets and treatments. Total and phosphor-ERK1/2 levels were determined by western blot analysis in SD rats fed with different salt diets at the end of the experiments. The p-ERK1/2/t-ERK1/2 ratio is presented here as a relative change in different groups in reference to that in the NS group. Note: \* $p < 0.05$  compared to the NS group; # $p < 0.05$  in the L+S group or in H+A compared to the LS group and the L+S group

performed siRNA knockdown experiments in HEK293 cells. In HEK293 cells transfected with either siRNA WNK4 or siRNA scramble control, we found that the knockdown of WNK4 expression significantly decreased ERK1/2 phosphorylation (Fig. 5b and d). These results suggest that WNK4 is the up-stream of the MAPK ERK1/2 signaling pathway.

#### Effect of aldosterone treatment on ERK1/2 phosphorylation in HEK293 cells

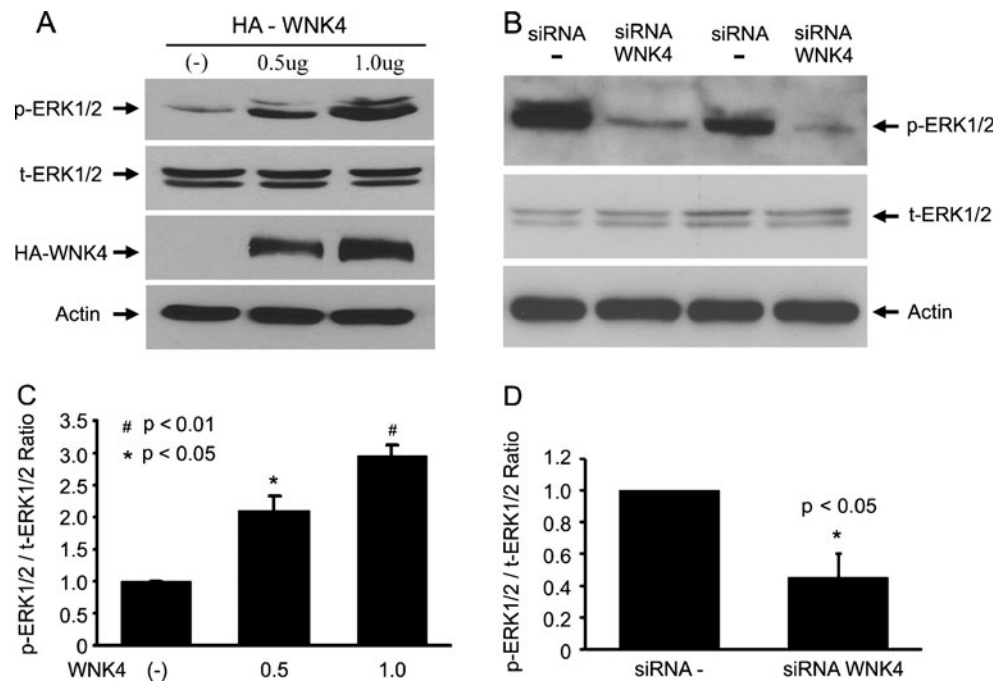
To further confirm whether aldosterone treatment directly affects ERK1/2 phosphorylation, we incubated HEK293 cells with aldosterone (1  $\mu$ M) for 3 h and then determined ERK1/2 phosphorylation by western blot analysis. As shown in Fig. 6, aldosterone treatment significantly enhanced ERK1/2 phosphorylation without affecting the total ERK1/2 level, indicating that aldosterone-mediated MAPK ERK1/2 signaling pathway exists in the cells.

## Discussion

WNK kinase has been found to participate in the regulation of ion transporters and channels, such as NCC. Dietary salt intakes and aldosterone have been shown to play an important role in the regulation of NCC function and protein expression. Emerging evidence demonstrated that WNK4 is implicated to be part of a novel signaling pathway mediating the regulation of NCC by dietary salt and aldosterone [24]. In the present study, we found that changes of dietary salt affect WNK4 and NCC mRNA and their protein expressions. Aldosterone increases NCC expression through altering WNK4-mediated ERK1/2 phosphorylation.

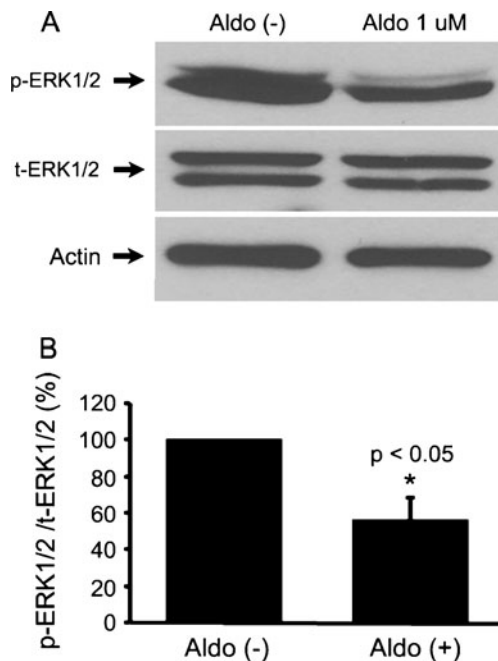
In the present study, after 2 weeks of dietary salt modifications, plasma aldosterone level increased, and urinary sodium excretion decreased in LS-fed rats, whereas in HS-fed rats, plasma aldosterone level decreased, while urinary sodium excretion increased. In addition, in LS-fed rats, urinary potassium excretion increased, whereas in HS-fed rats, urinary potassium excretion decreased. When HS-fed rats were given aldosterone infusion, blood pressure was markedly elevated, and urinary potassium excretion was significantly increased. These data suggest that aldosterone is involved in normal physiological regulation in response to dietary salt changes. The data obtained from our animal models are consistent with those obtained from a similar study [19]. Low dietary salt and aldosterone can up-regulate NCC mRNA and protein expression. It remains unclear what exact mechanism underlies this regulation. WNK4 WT was found to inhibit NCC function and its protein expression, whereas WNK4

**Fig. 5** Effect of WNK4 over-expression and knockdown of WNK4 expression on ERK1/2 phosphorylation in HEK293 cells. HEK293 cells were transiently transfected with either CD4 as control or two doses of WNK4 Wt (for panel a) or either siRNA scramble control (siRNA-) or siRNA WNK4 Wt (for panel c). Forty-eight hours after transfection, cells were lysed. Western blot analyses were performed to determine the levels of phosphor (p)-ERK1/2, total (t) ERK1/2, myc-WNK4 Wt and actin. The *top panel* shows representative western blot (a and c), and the *bottom panel* shows the summary data from the aforementioned three independent experiments (b and d). Note: \* $p < 0.05$ ; # $p < 0.01$  compared to control group



PHA II mutant increases NCC expression. WNK1 itself does not affect NCC function, but it prevents WNK4 from inhibiting NCC protein expression, ultimately up-regulating NCC function [31]. A previous study reported that NCC

mRNA expression was not changed, while NCC abundance increased in rats in response to short dietary salt restriction (1 or 3 days) [10], suggesting that a change in NCC abundance to early salt restriction is not a result of altered NCC transcription mediated by aldosterone. However, in the present study, when rats were given low-salt diet for a much longer time (2 weeks), increased NCC abundance in response to dietary salt restriction may be caused by the altered NCC gene expression.



**Fig. 6** Effect of aldosterone treatment on ERK1/2 phosphorylation in HEK293 cells. HEK293 cells were treated with aldosterone (1  $\mu$ M) for 3 h, and western blot analysis was then performed to determine the levels of phosphor (p)-ERK1/2, total (t)-ERK1/2 and actin. The *top panel* shows representative western blot (a), and the *bottom panel* shows the summary data from aforementioned three independent experiments (b). Note: \* $p < 0.05$  compared to control (aldo -) group

WNK1 was found to phosphorylate SPAK/OSR1 kinase and subsequently phosphorylate the threonine residues at the N-terminus of NCC, activating its activity [16]. WNK4 was also found to phosphorylate SPAK/OSR1 [23]. These findings suggest that WNK1 and WNK4 confer stimulatory regulation of NCC. Dietary salt was found to regulate the phosphorylation of SPAK/OSR1 kinase and NCC in mice, and aldosterone reversed the HS-mediated inhibition of NCC via increasing SPAK/OSR1 phosphorylation, suggesting that aldosterone enhances NCC expression through activating SPAK/OSR1 kinases [2]. However, the body of evidence demonstrated that WNK4 inhibits NCC function and its protein expression [1, 26, 29]. A recent study reveals that angiotensin II stimulates NCC activity through WNK4 and SPAK kinases [18], indicating that WNK4–SPAK signaling stimulates NCC activity. Our present data suggest that there is an alternative WNK4 signaling pathway participating in NCC regulation in response to dietary salt changes. The WNK4-ERK1/2 pathway that is involved in the decreased expression of NCC may be acting in a complimentary manner to control NCC activity with the WNK4/SPAK pathway. It remains to

be established how WNK4 modulates NCC under the different physiological conditions either through a stimulatory SPAK/OSR1 signaling pathway or through an inhibitory ERK1/2 signaling pathway or both.

Dietary salt changes have been shown to affect NCC protein abundance [19]. Aldosterone also up-regulates NCC protein expression. In the present study, we also found that low-salt diet increased NCC expression while significantly decreasing WNK4 expression in rats, whereas high-salt diet decreased NCC expression while increasing WNK4 expression. O'Reilly et al. also reported that in mice fed with LS diet for 6 days, NCC abundance is increased and WNK4 expression is decreased even though it does not reach statistical difference [13]. This may be due to the difference from dietary salt treatment. In our study, we used HS diet of 4% NaCl and LS diet of 0.07% NaCl, whereas O'Reilly and her colleagues used HS diet of 3% NaCl and LS diet of 0.03% NaCl. These data suggest that dietary salt change modulates the NCC protein expression through altering WNK4 expression since WNK4 has been shown to modulate NCC protein expression *in vitro* [1, 5, 29]. In LS-fed rats, plasma aldosterone level was increased, and NCC expression was up-regulated, whereas in the presence of spironolactone, NCC expression enhanced by elevated aldosterone was reversed in LS-fed rats. By adding aldosterone infusion in HS-fed rats, the urinary potassium excretion was increased, and NCC protein expression was also increased, while WNK4 expression decreased. These data indicate that aldosterone is likely an up-stream regulator involved in WNK4-mediated regulation of NCC. Our data are consistent with the recent report that aldosterone mediates activation of NCC activity through SGK1 and WNK4 signaling pathway in *Xenopus* oocyte expression system [17]. Taking all together, our data strongly suggest that WNK4 is involved in the inhibitory regulation of NCC in response to dietary salt change.

Chiga et al. reported that in wild-type mice, the phosphorylation states of NCC and OSR1/SPAK were increased by LS diets and decreased by HS diets, while this regulation was completely lost in the WNK4 PHA II mutant knock-in mice. These data indicate a stimulatory signaling pathway involving regulation of NCC [2]. They also showed that WNK4 protein level was unchanged by different levels of dietary NaCl [2], which is different from our finding in normal rats in response to dietary salt changes. Although we used same dietary salt concentrations in the present study as they did, tissue lysate preparation was different, in which the kidney tissue homogenate in the present study was not centrifuged to remove crude membrane fraction, whereas Chiga and his colleagues made tissue homogenate by centrifugation (17,000 g) to remove crude membrane fraction. Since WNK4 was shown to be located not only in the cytoplasm but in intercellular

junctions in the cortical collecting duct [25], whether the removal of crude membrane fraction that likely includes the fraction of intercellular junctions would contribute to the difference of total WNK4 protein abundance remains to be further investigated.

This evidence, including ours, suggests that WNK4 might have more sophisticated roles in the regulation of NCC than what we thought before. WNK4 was shown to phosphorylate ERK1/2 in HEK293 cells [20]. The phorbol ester was shown to increase the phosphorylation of ERK1/2 and ultimately inhibit NCC activity [7]. We also found that LS diet increased NCC expression that was associated with the decrease in ERK1/2 phosphorylation level *in vivo*, whereas HS diet inhibited NCC expression that was correlated with the increase in ERK1/2 phosphorylation. Furthermore, LS diet suppressed WNK4 expression and decreased ERK1/2 phosphorylation, ultimately increasing NCC expression. These data suggest that different salt diets change NCC expression through altering ERK1/2 phosphorylation. In addition, we further showed that over-expression of WNK4 enhances ERK1/2 phosphorylation in a dose-dependent manner, whereas knockdown of WNK4 expression decreases ERK1/2 phosphorylation in HEK293 cells. Aldosterone treatment enhanced ERK1/2 phosphorylation in HEK293 cells. Taken all together, our data suggest that a novel aldosterone-mediated WNK4-ERK1/2-NCC inhibitory signaling pathway is likely present, involving NCC regulation in response to dietary salt changes. We have previously demonstrated that WNK4 inhibits NCC protein expression by enhanced NCC degradation through a lysosomal pathway [1, 32]. The WNK4/ERK1/2 signaling pathway may trigger this degradation process. Further exploration is needed to provide the detailed mechanisms underlying this process.

The kidney plays an important role in maintaining water and salt homeostasis. NCC, exclusively expressed in distal convoluted tubule, is responsible for 5–10 % of sodium reabsorption. Aldosterone and dietary salt modifications will affect renal sodium excretion through renal sodium transporter or ion, such as NCC, and subsequently affect blood pressure regulation. Our present study demonstrated that WNK4 kinase plays an important role in the regulation of NCC likely through a novel WNK4-ERK1/2 signaling pathway *in vivo*, and this WNK4-ERK1/2 signaling pathway is negatively modulated by aldosterone. Further study is needed to determine how WNK4 modulates NCC under different physiological conditions involving either ERK1/2 signaling pathway or WNK4–SPAK signaling pathway.

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