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ION CHANNELS, RECEPTORS AND TRANSPORTERS

# NKCC2 is activated in Milan hypertensive rats contributing to the maintenance of salt-sensitive hypertension

**Monica Carmosino · Federica Rizzo · Patrizia Ferrari · Lucia Torielli · Mara Ferrandi · Giuseppe Bianchi · Maria Svelto · Giovanna Valenti**

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**Abstract** The Milan hypertensive strain of rats (MHS) develops hypertension as a consequence of the increased tubular $Na^+$ reabsorption sustained by enhanced expression and activity of the renal tubular Na–K–ATPase. To verify whether the Na–K–2Cl cotransporter (NKCC2) is involved in the maintenance of hypertension in MHS rats, we have analysed the phosphorylation state and the activation of NKCC2 in Milan rats. Western blotting and immunofluorescence experiments were performed using specific antibodies against the regulatory phospho-threonines in the NKCC2 N terminus (R5 antibody). The phosphorylation levels of NKCC2 were significantly increased in the kidney of MHS rats. Moreover, the administration of furosemide in vivo decreased the blood pressure and increased the urine output and natriuresis in MHS rats demonstrating the actual involvement of NKCC2 activity in the pathogenesis of hypertension in this strain of rats. The up-regulation of NKCC2 activity is most probably mediated by a STE20/SPS1-related proline/alanine-rich kinase (SPAK) phosphorylation at serine-325 since it was significantly increased in MHS rats. Interestingly, aldosterone treatment caused an increase in NKCC2 phosphorylation in NKCC2-expressing MDCK cells. In conclusion, we demonstrated an increase in the activity of NKCC2 along the TAL that significantly contributes to the increase in systemic blood pressure in MHS rats. The elevated plasma levels of aldosterone, found in MHS rats, may influence $Na^+$ balance through a SPAK-dependent regulation of NKCC2 accounting for the maintenance of the hypertensive state in MHS rats.

**Keywords** Na+/K+/2Cl cotransport · Phosphorylation · Hypertensive rats · Aldosterone · Furosemide

M. Carmosino (✉) · F. Rizzo · M. Svelto · G. Valenti
General and Environmental Physiology, University of Bari, Bari, Italy
e-mail: monica.carmosino@uniba.it

P. Ferrari · L. Torielli · M. Ferrandi
Prassis Research Institute, Sigma Tau, Milan, Italy

G. Bianchi
S. Raffaele Hospital, Università Vita Salute, Milan, Italy

## Introduction

Hypertension affects 1.5 billion people worldwide and causes 7.1 million deaths per year, making it the third cause of death in the world [46]. Regulation of arterial blood pressure is a complex phenomenon with many interacting genetic and environmental factors. Blood pressure is a function of cardiac output and peripheral resistance, which is influenced by extracellular fluid volume, and the kidneys play a major role in the long-term control of this volume by matching urinary $Na^+$ and water output to dietary intake. A variety of approaches have demonstrated that the inability to excrete $Na^+$ leads to increased blood pressure in humans and experimental animals. On intravenous infusion of 75 mEq/h saline, renal $Na^+$ excretion is markedly blunted in patients with essential hypertension [25]. Numerous studies also point to a causal link between a chronically high salt intake and the development of hypertension when the kidneys have a reduced ability to excrete salt [27]. Moreover, the cross-transplantation of kidneys between normotensives and hypertensives has provided strong evidence that the kidney plays a key role in primary hypertension both in humans and rats [4, 21].

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One model of genetic hypertension is represented by the Milan hypertensive rat strain (MHS) that develops hypertension because of a primary alteration in renal tubular $Na^+$ reabsorption [3], linked to increased activity and expression of Na–K–ATPase [16, 26] associated with a point mutation of adducin [43], a cytoskeletal protein involved in actin polymerization and cell signal transduction [22]. This mutation causes a deficient endocytosis of the sodium pump that, by affecting the time that this molecule resides in the plasma membrane, may be an important contributing factor for the increased capacity of the renal tubule cells to reabsorb sodium [13, 42]. $Na^+$ reabsorption across the nephron is controlled by the driving force of the Na–K–ATPase, which is coupled with the $Na^+$ entry across the apical membrane. Therefore, any change in the quantity and/or activity of the proteins mediating apical $Na^+$ entry should affect the reabsorption rate. The expression levels of the most important apical membrane transporters expressed in the kidney of Milan rats have been analysed. It has been demonstrated that $Na^+$ entry is increased during the induction phase of hypertension in young MHS rats, paralleled by an up-regulation of the Na–K–2Cl cotransporter (NKCC2) at the level of the thick ascending limb (TAL) suggesting a key role of this cotransporter in the genesis of hypertension in this rat model [5]. In contrast, the expression of the NKCC2 in the TAL of adult Milan rats with established hypertension resulted unchanged compared to the age-matched control rats [6]. Moreover, in the adult MHS animals, the apical Na–Cl transporter (NCC) expressed in distal convoluted tubuli (DCT) is up-regulated, thus identifying this segment of the nephron as the possible site responsible for the maintenance phase of hypertension in this strain of rats [6]. While the authors suggested that the up-regulation of NCC could potentially contribute to the significant rise in blood pressure observed in MHS, they did not exclude the implication of other $Na^+$ transporters in this process. Among them, here, we focused our attention on the phosphorylated form of NKCC2, which represents the active form of this crucial cotransporter. Interestingly, previous data indicated that the NKCC2-mediated $^{86}Rb^+$ flux is functionally up-regulated in adult hypertensive MHS as compared with age-matched MNS controls [15]. Therefore, despite NKCC2 protein and mRNA expression not being increased in adult MHS, it appears relevant to investigate the phosphorylation and the functional activation of this cotransporter in this strain of rats. The central role of the NKCC2 activity in the vectorial transepithelial salt reabsorption along the loop of Henle is evidenced by the effects of loop diuretics, the pharmacological inhibitors of NKCC2, that are among the most powerful antihypertensive drugs available to date. Moreover, loss-of-function genetic mutations of the NKCC2 encoding gene resulted in a severe phenotype known as type I Bartter's syndrome, characterized by a severe volume depletion, hypokalemia, and metabolic alkalosis with high prenatal mortality [36]. In this work, we investigated for the first time the phosphorylation levels of NKCC2 in the Milan rats to evaluate its possible functional involvement in the maintenance of the hypertensive state in this strain of rats. Intracellular chloride depletion activates NKCC2 by promoting the phosphorylation of three highly conserved threonines (96, 101, and 111) in the amino terminus. The chloride-sensitive activation of NKCC2 requires its interaction with two serine–threonine kinases, with-no-lysine kinase (WNK3) and a STE20/SPS1-related proline/alanine-rich kinase (SPAK). SPAK appears to be the chloride-sensitive kinase downstream of WKN3 and the direct activator of NKCC2 [31]. We analysed the phosphorylation state and the activity of the cotransporter in Milan rats and the possible elements of the regulatory machinery involved in this process.

## Methods

### Antibodies

NKCC2 was detected with a T4 monoclonal antibody (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA). The phosphorylated form of NKCC2 was detected using rabbit R5 polyclonal antibody raised against a diphosphopeptide containing Thr (212) and Thr (217) of human NKCC (kindly provided by Dr. B. Forbush, Yale University, New Haven, CT). The sheep anti-mouse SPAK and the sheep anti-human SPAK phospho-serine-373 (corresponding to phospho-serine-325 in rats) antibodies were from the Division of Signal Transduction Therapy, University of Dundee, Scotland.

### Animal care

The care and husbandry of rats complied with European Directive no. 86/609 and with the Italian Law (DL116; January 27, 1992). The authorization for animal use in Prassis Sigma Tau laboratories was obtained from the Italian Health Authority. Rats had free access to tap water and food and were maintained under controlled conditions of air temperature (22±2°C), relative humidity (50±5%) and day/night cycle (12 h). Rats were sacrificed by cervical dislocation after inducing ether anaesthesia.

### Immunofluorescence

Kidneys from Milan rats were fixed in 4% paraformaldehyde in PBS and then cryoprotected by incubation

overnight in 30% sucrose in PBS. Sections of 5 μm were cut and mounted on Superfrost Plus Glass. After three washes in PBS, sections were blocked in saturation buffer (1% bovine serum albumin in PBS) for 20 min at room temperature (RT) and incubated with the primary antibodies for 2 h at RT (R5, 1:1,000; SPAK, 1:100; phosphorylated (p)-SPAK, 1:100) in saturation buffer. After three washes in PBS, sections were incubated with the appropriate Alexa Fluor conjugated secondary antibodies for 1 h at RT. Confocal images were obtained with a laser scanning fluorescence microscope Leica TSC-SP2.

### Tissue homogenization and fractionation

Whole rat kidneys were homogenized in ice-cold anti-phosphatase buffer (150 mM NaCl, 30 mM NaF, 5 mM EDTA, 15 mM $Na_2HPO_4$, 15 mM pyrophosphate, and 20 mM HEPES, pH 7.2, containing 1% Triton X-100 and 0.5 μM calyculin A) using 25 strokes at 25,000 rpm with a Polytron tissue homogenizer and centrifuged for 10 min at 4,000×*g* at 4°C to pellet debris. The supernatant was centrifuged for 40 min at 20,000×*g* at 4°C, and the resulting membrane-containing pellet was resuspended in anti-phosphatase buffer. Membranes were then used for Western blotting experiments using R5 and T4 antibodies.

### SPAK immunoprecipitation experiments

Kidneys of Milan rats were homogenized in ice-cold anti-phosphatase buffer with 1% Triton X-100 and 0.5 μM calyculin A using 25 strokes at 25,000 rpm with a Polytron tissue homogenizer. After 30 min of lysis on ice, the lysates were clarified by centrifugation at 13,000×*g* for 30 min at 4°C. The lysates (500 μg) were pre-cleared with 100 μl of protein A-Sepharose suspension (Sigma) for 1 h at 4°C and then incubated with 2.5 μg of sheep polyclonal antibody to SPAK overnight at 4°C under rotation. Immunocomplexes were bound using 100 μl of protein A-Sepharose suspension for 2 h at 4°C under rotation and then washed five times with anti-phosphatase buffer. Immunocomplexes were dissociated in NuPAGE LDS sample buffer (Invitrogen) with 100 mM DTT, heated at 95°C for 10 min and resolved on gel. After transfer to the Immobilon P membrane, lanes were probed with antibodies against either SPAK (1:1,000) or p-SPAK (1:1,000).

### Chronic furosemide administration and blood pressure measurements

Four-week-old MHS rats, weighting 110–120 g, were orally treated for 6 weeks with 20 mg/kg/day furosemide dissolved in 0.5% Methocel ($n$=7), while MHS controls ($n$=7) received only a vehicle (0.5% Methocel).

Systolic blood pressure (SBP) and heart rate (HR) were recorded weekly at the tail by plethysmography (BP recorder, U. Basile, Varese, Italy) in conscious rats. Before each measurement, the rats were trained to be manipulated by the same operator. The rats were pre-warmed at 37°C for 15 min in thermostatic boxes; afterwards, they were gently wrapped for SBP and HR measurements. Eight to ten consecutive inflation–deflation cycles were performed on each rat, and the mean of the last five recordings was taken as the final value of SBP and HR.

### Acute furosemide treatment and urinary parameters

The rats were acclimatized in individual metabolic cages for 2 days and had free access to tap water and food. On the third day, the rats received one single dose of furosemide (60 mg/kg, dissolved in 0.5% Methocel) by oral gavage, and control rats received only the vehicle (Methocel). The rats have been previously demonstrated to be responsive to this dose of furosemide [23].

Urine samples were collected 6 and 24 h after drug treatment. Urine samples were centrifuged at 3,000 rpm for 20 min, and the supernatants were used for the determination of $Na^+$ concentration (flame photometry: IL943, Instrumental Laboratories, Milan, Italy) and urinary creatinine (enzymatic measurement, the kit from Sentinel Diagnostics, Milan, Italy). Urinary aldosterone concentrations were determined using a commercially available radioimmunoassay kit (ALDOCTK-2 dia Sorin cod. P2714, Italy).

### Plasma aldosterone measurements

The rats were anaesthetized by ether, the abdomen was opened, and blood was drawn from the abdominal aorta. Blood collection was interrupted as soon as dyspnoea appeared. Plasma aldosterone concentrations were determined using a commercially available radioimmunoassay kit (ALDOCTK-2 dia Sorin cod. P2714, Italy).

### Aldosterone treatment of MDCK cells in culture

Madin Darby canine kidney (MDCK) cells, stably transfected with a functional NKCC2 construct [7], were treated with 100 nM aldosterone (Sigma), in the presence or absence of 100 nM spironolactone (Sigma), in a culture medium for either 2 or 4 h. The cells were then lysed in anti-phosphatase buffer, and the lysates were assessed by Western blotting for p-NKCC2 expression using the R5 antibody.

## Results

### Expression of p-NKCC2 in adult Milan rats

As shown in Fig. 1a, the R5 antibody raised against two conserved phospho-threonines in the NKCC2 N terminus [19] recognized in kidney membranes from adult Milan rats a broad band centred at about 160 kDa corresponding to the fully glycosylated form of NKCC2. The signal corresponding to phosphorylated NKCC2 was clearly increased in MHS rats compared to age-matched normotensive MNS rats (Fig. 1a, p-NKCC2). We then verified the expression of total NKCC2 using T4 antibody, which recognizes both the fully glycosylated (gly) and unglycosylated forms (un-gly) of NKCC2 (Fig. 1a, total (t)-NKCC2). Interestingly, Western blotting using this antibody revealed that the abundance of total NKCC2 in MHS rats was unchanged compared to that in MNS rats in agreement with a previous report [6]. The densitometric analysis of the p-NKCC2 band, normalized to the corresponding glycosylated form of the t-NKCC2 band, showed that the phosphorylation of NKCC2 increased by about 58% in MHS rats (Fig. 1a densitometry).

Kidneys from the same rats were used for immunofluorescence experiments. Figure 1b shows p-NKCC2 immunofluorescence staining of tissue sections. Both the number of stained tubules and the signal intensity of single tubules were much higher in the renal cortex of MHS than in MNS rats. These results confirm and reinforce the Western blot data, indicating that the phosphorylation of NKCC2 is up-regulated in adult MHS rats.

### Effects of chronic administration of furosemide on blood pressure in adult MHS rats

It has been demonstrated that the reactivity of the R5 antibody with NKCC directly mirrors their functional

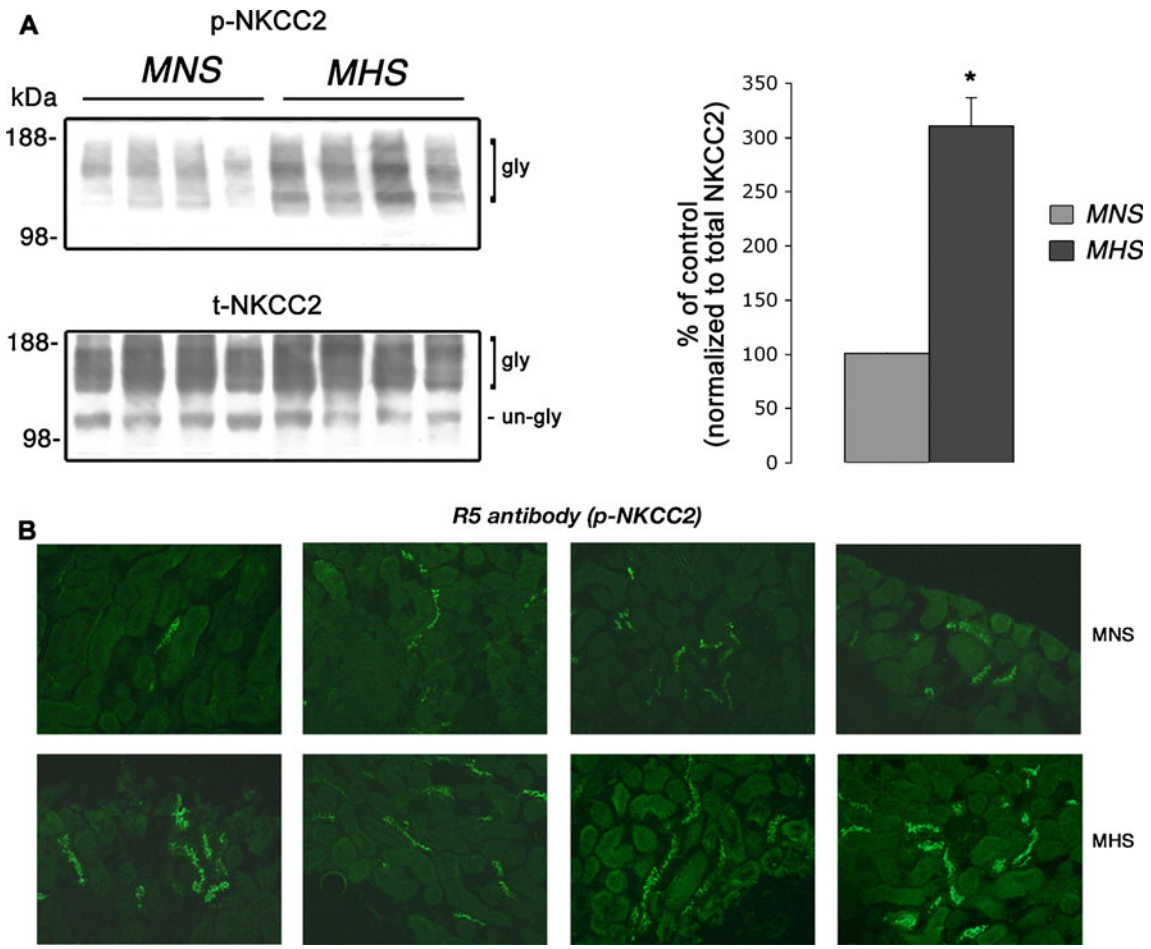


**Fig. 1** **a** Western blotting analysis of phosphorylated NKCC2 (*p-NKCC2*) and total NKCC2 (*t-NKCC2*) in Milan rat kidney medullae. The p-NKCC2 immunoreactivity significantly increased in hypertensive MHS rats compared to that of age-matched MNS rats. In contrast, the total amount of NKCC2 was unchanged in both strains of rats. The densitometric analysis of the p-NKCC2 band normalized to t-NKCC2 showed an increase of about 58% in NKCC2 phosphorylation in MHS (densitometry). These data are representative of three independent experiments. Student's *t* test, $*P<0.0001$. **b** Immunofluorescence analysis of p-NKCC2 in Milan rat kidney sections. Both the number of stained tubules and the signal intensity of single tubules were much higher in the renal cortex of MHS than in MNS rats

activation as assayed by $^{86}Rb^{+}$ fluxes in different cell lines [19]. Thus, we can conclude that the increase in the extent of NKCC2 phosphorylation observed in MHS rats coincides with an increase in NKCC2 activation that in turn might be implicated in the maintenance of hypertension in this strain of rats. To verify this possibility, two groups of MHS rats have been treated with either 20 mg/kg/day of furosemide, a specific NKCC2 inhibitor, or the vehicle alone for 6 weeks by oral administration starting at the first month of life. After 6 weeks of treatment, the blood pressure of furosemide-treated MHS rats was significantly lower than that of the vehicle-treated MHS controls clearly demonstrating that NKCC2 contributes to the maintenance of hypertension in this strain of rats (Table 1).

## Effects of acute administration of furosemide on urinary parameters in adult MHS rats

Milan rats were treated with a single oral dose of 60 mg/Kg of furosemide, and urinary volume and natriuresis were measured. MHS rats had a slightly but significantly higher diuresis compared to MNS rats, in agreement with a previous report [3]. However, furosemide treatment resulted in a stronger increase in urinary volume in MHS rats compared to MNS rats (Fig. 2a). In addition, the 24-h $Na^{+}$ excretion normalized to creatininuria was significantly higher after furosemide treatment in MHS rats compared to MNS rats (Fig. 2b). The greater sensitivity to furosemide suggests that NKCC2 activity is up-regulated in MHS rats.

## Expression of p-SPAK in adult Milan rats

The activation of NKCC2 requires its interaction with the two serine–threonine kinases, WNK3 and SPAK. SPAK appears to be the kinase downstream of WKN3 and the direct activator of NKCC2 [31]. We then analysed the expression and activation state of SPAK kinase in MHS and MNS rats to tentatively identify elements of the intracellular machinery involved in the activation of NKCC2 observed in the MHS strain.

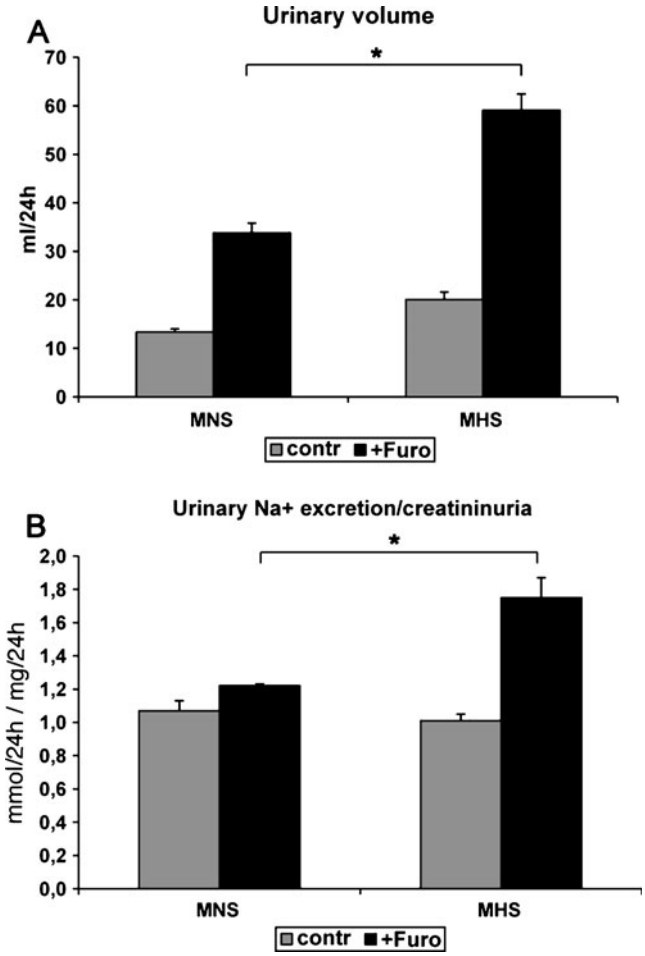


**Fig. 2** Urine output and sodium excretion before (*contr*) and after (+*Furo*) acute furosemide administration in both MNS and MHS rats. Furosemide treatment resulted in a stronger increase in urinary volume and natriuresis in MHS compared to MNS rats. $n=10$; Student's $t$ test, $*P<0.0001$

To test for SPAK expression and function, we immunoprecipitated the kinase from renal extracts and performed Western blotting using antisera specific for total or for phosphorylated (active) variants of the kinase. Specifically, we followed the phosphorylation state of the serine-325 in the C-terminal tail of SPAK, corresponding to the critical serine-

**Table 1** Blood pressure measurement in MHS rats before (ctr) and after (+ furo) the chronic administration of furosemide. Furosemide prevented the increase in blood pressure in MHS rats

| | Sixth week of treatment | | |
|---|---|---|---|
| | BW (g) | SBP (mmHg) | HR (beats/min) |
| MHS ctr $n=7$ | 300±11 | 161±2 | 350±6 |
| MHS + furo 20 mg/kg $n=7$ | 282±7 | 148±4* | 333±7 |
| | | $P<0.01$ | n.s. |

*The difference is statistically significant, $P<0.01$

n.s. means not significant

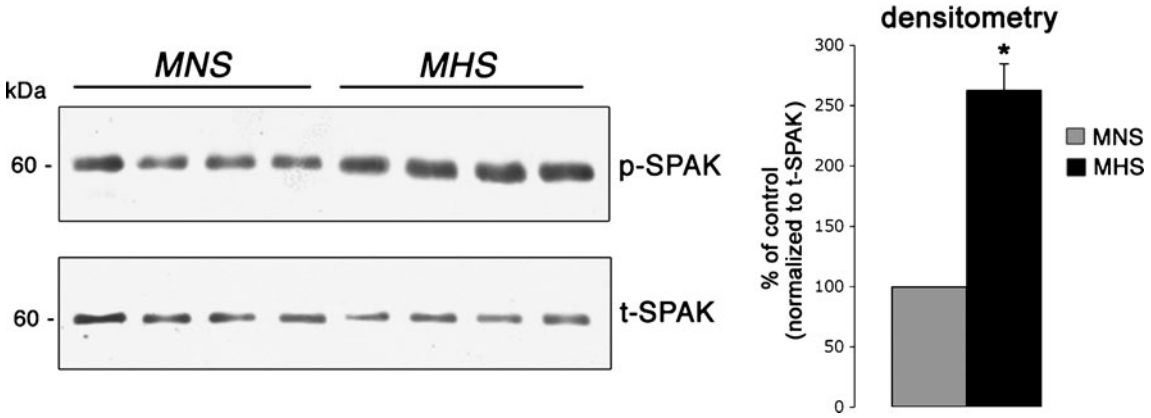


**Fig. 3** Western blotting analysis of phosphorylated SPAK kinase at serine-325 (*p-SPAK*) and total SPAK kinase (*t-SPAK*) immunoprecipitated from Milan rat kidney lysates. A significant increase in p-SPAK but not in t-SPAK was seen in MHS rats. Densitometric scanning of the p-SPAK Western blot band normalized to the total SPAK band showed an increase in SPAK phosphorylation of about 50% in MHS rats (densitometry). These data are representative of three independent experiments. Student's *t* test, $*P<0.0001$

373 in human SPAK. Intriguingly, a clear increase in p-SPAK, but not in total SPAK (t-SPAK) was seen in MHS rats (Fig. 3).

Densitometric analysis of the p-SPAK Western blot band normalized to the t-SPAK band showed a significant increase of about 50% of p-SPAK in MHS versus MNS rats (Fig. 3, densitometry). Elevated levels of p-SPAK in MHS were further substantiated by immunohistological analysis (Fig. 4). p-SPAK immunoreactivity (green) is clearly stronger in the kidney of MHS rats. Moreover, colocalization experiment with p-NKCC2 (red) clearly demonstrated that the p-SPAK is mainly localized in the TAL in both strains of rats (yellow). Few tubules resulted positive only for p-SPAK in both rat strains likely corresponding to distal convoluted tubuli.

## Measurement of aldosterone in the plasma and urine of Milan rats

Aldosterone levels in both the plasma and urine of the Milan rats used in our study are reported in Table 2. The plasma level of aldosterone was found to be significantly higher in MHS

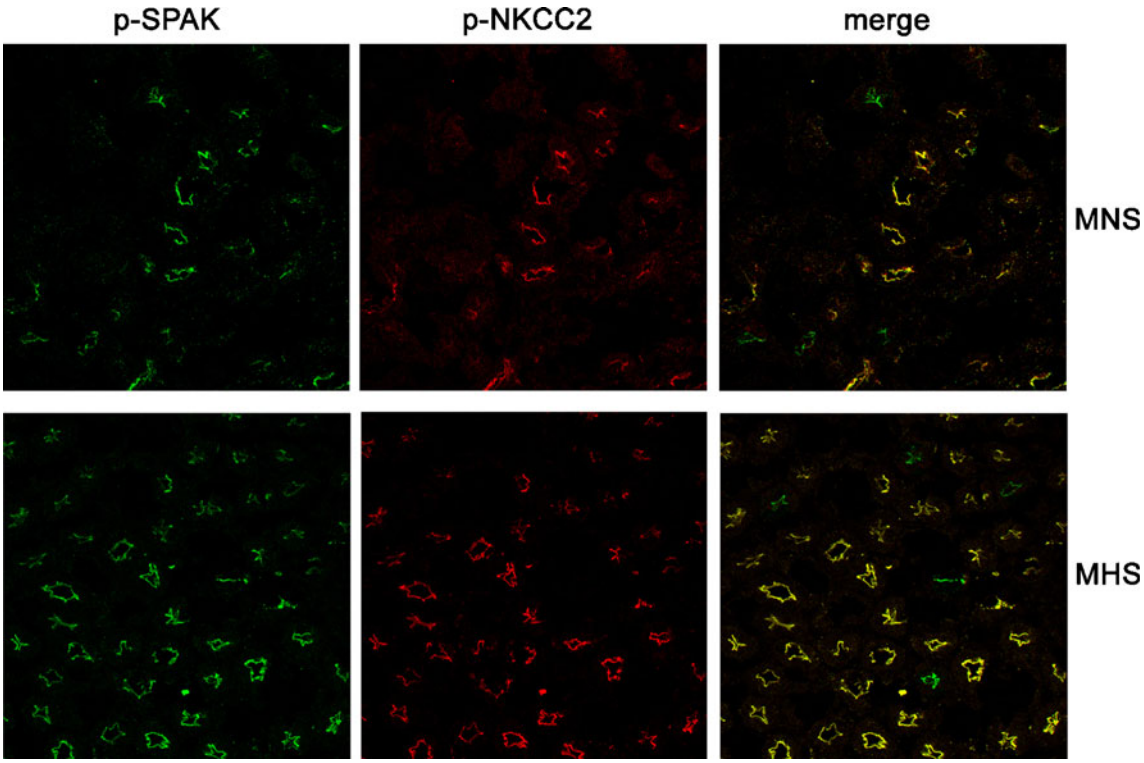


**Fig. 4** Immunofluorescence analysis of p-SPAK and p-NKCC2 in Milan rat kidney sections. Both the p-SPAK staining (*green*) and p-NKCC2 staining (*red*) were higher in the renal cortex of MHS than in MNS rats. Moreover, both signals overlap in the TAL (*merge*, *yellow*)

**Table 2** Measurement of plasma and urinary levels of aldosterone in Milan rats

| | Plasma level of aldosterone (ng/dl) | Urinary level of aldosterone (ng/24 h) |
|---|---|---|
| MNS | 4±1 ($n$=6) | 12±1 ($n$=8) |
| MHS | 11±2* ($n$=6) | 28±4* ($n$=8) |

Both values resulted significantly higher in MHS than in MNS rats
*$P$<0.01

rats compared to MNS rats. These data confirmed previous reports [6]. Accordingly, the urinary level of aldosterone in the 24-h urine sample was significantly increased in MHS rats compared to the age-matched control rats. This finding may suggest that the elevated level of aldosterone in MHS rats may be responsible for the up-regulation of NKCC2.

## Effect of aldosterone on NKCC2 phosphorylation in intact cells

To verify whether aldosterone treatment causes an increase in NKCC2 phosphorylation, we performed in vitro experiments using MDCK cells stably transfected with a functional and characterized construct of NKCC2 [7, 8].

Interestingly, strong mineralocorticoid receptor (MR) mRNA and protein expression in the TAL have been previously reported [1, 14] suggesting that NKCC2 in the TAL cells can actually be the target of aldosterone.

MDCK cells were incubated for either 2 or 4 h with 100 nM aldosterone in a culture medium and then lysed in anti-phosphatase buffer. The lysates were assessed for the expression of p-NKCC2 by Western blotting. As shown in Fig. 5 A, the signal relative to p-NKCC2 significantly increased after either 2 or 4 h of aldosterone treatment, compared to control conditions. Moreover, the expression level of t-NKCC2 was unchanged in all the experimental conditions demonstrating that the effect of aldosterone was not related to an increase in NKCC2 protein synthesis. The densitometric analysis of the p-NKCC2 band normalized to the t-NKCC2 band showed that NKCC2 phosphorylation increased by about 50% in aldosterone-treated cells (Fig. 5 A′).

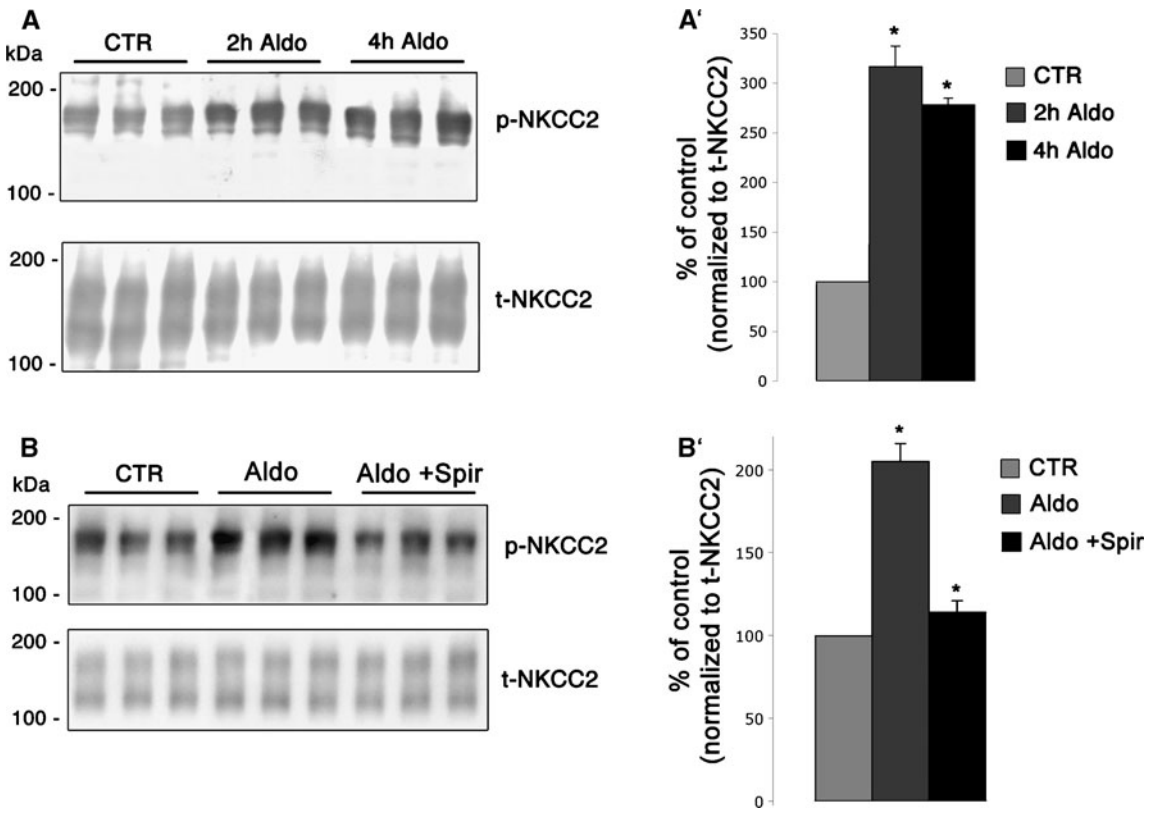


**Fig. 5** *A* Western blotting analysis of phosphorylated NKCC2 (*p-NKCC2*) and total NKCC2 (*t-NKCC2*) in NKCC2-transfected MDCK cells treated with 100 nM aldosterone. The immunoreactivity of p-NKCC2 was significantly higher in aldosterone-treated MDCK cells. In contrast, the expression level of t-NKCC2 was unchanged in all the experimental conditions. *A′* The densitometric analysis of p-NKCC2 normalized to t-NKCC2 showed that p-NKCC2 increased by about 50% higher after either 2 or 4 h of aldosterone treatment. *B* Western blotting analysis of phosphorylated NKCC2 (*p-NKCC2*) and total NKCC2 (*t-NKCC2*) in NKCC2-transfected MDCK cells treated with 100 nM aldosterone+100 nM spironolactone for 2 h. In this experimental condition, the increase in NKCC2 phosphorylation was prevented. *B′* The densitometric analysis of p-NKCC2 normalized to t-NKCC2. $P$>0.001

To verify the specificity of aldosterone in MDCK cells, 2 h of aldosterone treatment was performed in the presence of 100 nM spironolactone, a specific antagonist of aldosterone. Interestingly, under this experimental condition, the increase in NKCC2 phosphorylation was prevented demonstrating that aldosterone actually acts through mineralocorticoid receptors in MDCK cells (Fig. 5 B, B′).

## Discussion

In this work, we analysed for the first time the phosphorylation and activation state of NKCC2 in adult MHS rats. Our results established that the NKCC2 activity is significantly up-regulated in the TAL of adult MHS rats identifying NKCC2 as a crucial player in the maintenance of this form of salt-sensitive hypertension. We found that the mechanism does not rely on the increases in NKCC2 mRNA and protein levels, in agreement with previous data [6] but is mediated by NKCC2 phosphorylation and consequent cotransporter activation.

Using the R5 antibody, selectively raised against the regulatory phospho-threonines in the N-terminal tail of NKCC2, we demonstrated that the phosphorylation state of the cotransporter was increased by about twofold in hypertensive MHS rats compared to age-matched normotensive MNS rats. Moreover, the chronic administration of furosemide in vivo revealed that the increase in blood pressure in MHS rats was prevented suggesting a possible involvement of NKCC2 in the maintenance of the hypertension in this strain of rats. Of note, the larger natriuresis and diuresis observed in MHS rats after acute furosemide administration convincingly demonstrated that NKCC2 is implicated in the pathogenesis of salt-sensitive hypertension in MHS rats.

Our data on the overactivity of NKCC2 in MHS rats are in agreement with the observation that in microsomes from adult MHS rats, the rate of NKCC2-mediated $^{86}Rb^{+}$ flux appeared to be significantly greater than in MNS control rats [15]. Moreover, this effect reflects an increased $^{86}Rb^{+}$ affinity of the cotransporter rather than an increased cotransporter density, consistent with the cotransporter activation by phosphorylation.

Previous reports in other animal models of hypertension showed that NKCC2 activity might be increased by post-translation modifications rather than by an increase of gene expression. For instance, progression from pre-hypertensive to hypertensive state in spontaneously hypertensive rats (SHR) is accompanied by an increase in steady-state protein levels of NKCC2 and its distribution to plasma membrane compared to control rats [37, 38]. Interestingly, the same amount of NKCC2 mRNA has been found in both SHR and control rats suggesting that the NKCC2 overexpression was due to a greater translational efficiency or an enhanced membrane stability of the cotransporter in SHR rats.

In addition, TAL tubules from Dahl salt-sensitive (DS) rats, a model of salt-dependent hypertension, exhibited a significantly higher NKCC2 activity than the Dahl salt-resistant strain. Western blot analysis revealed that NKCC2 displayed a different pattern of glycosylation in DS rats, suggesting that NKCC2 might accumulate at different stages of its biosynthesis or degradation in these rats [2].

In this work, we also tried to identify the elements of the cellular machinery involved in the up-regulation of NKCC2 activity observed in Milan rats. Since the discovery that mutations within human WNK1 and WNK4 genes cause a form of hypertension and hyperkalemia termed Gordon's syndrome (pseudohypoaldosteronism type II, PHAII) [18], these enzymes have taken centre stage in research aimed at understanding signal transduction networks that regulate ion homeostasis and blood pressure.

The best characterized WNK substrates comprise SPAK kinase and the oxidative stress-responsive kinase 1 (OSR1), which are activated following their phosphorylation by WNK1 or WNK4 [11, 33].

These WNK isoforms interact directly with SPAK, as well as OSR1, and phosphorylate these enzymes at two conserved residues, namely the T-loop thr residue and the ser residue in the S-motif (ser-373 in humans and ser-325 in rats). For review, see [33].

Using yeast two-hybrid screens, SPAK was found to interact with the amino terminal domain of NKCCs [11, 30]. The ability of SPAK to act as NKCC kinase was further demonstrated in cell culture studies [12, 28, 33]. Recently, the relevance of SPAK for phosphorylation of several cation–chloride cotransporters was confirmed in mice lacking SPAK [20] or expressing a mutant variant of SPAK that cannot be activated [32]. Specifically, NKCC2 revealed a significant decrease in phosphorylation in SPAK mutant transgenic mice compared to wild-type mice [32].

On the other hand, recent reports showed a decrease in p-NCC and an increase in p-NKCC2 in SPAK knockout mice suggesting NCC as a unique target of SPAK kinase in the kidney. However, in these mice, the amount of both total NKCC2 and p-OSR1 are up-regulated, thus accounting for the increase in p-NKCC2 [47].

Here, we demonstrate that the phosphorylation of NKCC2 at the N-terminal threonines in the TAL of MHS rats is paralleled by an increase in the phosphorylation of SPAK at its critical serine-325 suggesting this kinase as possibly responsible for NKCC2 activation in these rats. Since the total amount of SPAK is unchanged in both hypertensive and normotensive Milan rats, an increase in SPAK gene transcription does not account for the increased level of SPAK phosphorylation.

MHS rats are characterized by a significant increase in aldosterone plasma level [6], due to mutations in the aldosterone synthase gene [24], which may contribute to blood pressure control in this strain of rats. We measured the plasma and urinary levels of aldosterone in the Milan rats used in this work confirming that they are up-regulated in MHS rats (Table 2). Accordingly, we previously demonstrated that the chronic treatment of MHS rats with carnenone, a specific aldosterone antagonist, induced a significant decrease in the blood pressure in MHS rats [17].

Aldosterone may act by up-regulating the SPAK-NKCC2 axis. Although the analysis of NKCC2 phosphorylation in MHS treated with the aldosterone antagonist spironolactone could better address the relationship between aldosterone and NKCC2 phosphorylation, many pieces of evidence support our hypothesis.

First, it has been demonstrated that the phosphorylation states of the critical SPAK serine (serine-325) was increased by low-salt diets and decreased by high-salt diets, and the increased SPAK phosphorylation was reproduced by the administration of exogenous aldosterone [10]. Second, in rats that underwent adrenalectomy and then received either aldosterone or a vehicle, it has been observed that aldosterone increased 6.1-fold the phosphorylation of SPAK at serine-325 compared to the control [44]. Together, these studies suggest that the cascade mediated by SPAK phosphorylation at serine-325/373 may be a novel effector system of aldosterone action in the kidney, which in turn might regulate NKCC2 activity.

Interestingly, strong mineralocorticoid receptor (MR) mRNA and protein expression in the TAL has been previously reported [1, 14] suggesting that NKCC2 in the TAL cells can actually be the target of aldosterone.

Moreover, several lines of evidence support this hypothesis. For instance, it has been previously reported that adrenalectomy reduces $Na^+$ reabsorption in the medullary thick ascending limb of rats, and chronic aldosterone treatment restores transport to normal [40, 45].

Then, aldosterone administration to adrenalectomized animals increases their ability to concentrate urine maximally, an action consistent with an effect on the thick ascending limb of Henle [34].

Of note, we demonstrated an increase in NKCC2 phosphorylation but not in NKCC2 expression in NKCC2-expressing MDCK cells stimulated with aldosterone, confirming that aldosterone can directly induce NKCC2 phosphorylation without changing the expression level of the cotransporter. The effects of aldosterone were independent of the transcription of new NKCC2 transporter units because, in the system used in this study, the transcription of NKCC2 was under control of the cytomegalovirus promoter that does not respond to steroids. On the other hand, NKCC2-transfected MDCK cells are sensitive to mineralocorticoids since MDCK cells express endogenous levels of functional mineralocorticoid receptors [29], which, therefore, can activate the pathways that lead to modification of the pre-existing NKCC2 units. Accordingly, we demonstrate that the effect of aldosterone in MDCK cells was specific since it was prevented by spironolactone, a selective aldosterone antagonist on mineralocorticoid receptors.

Our findings are in agreement with previous observations on the regulation of $Na^+$ transporters by aldosterone in its early phase of action in which it does not affect the synthesis of new $Na^+$ transporter units. The early, short-term action of aldosterone (lasting 2–4 h after the lag period) is considered to be largely mediated by induced regulatory proteins or transcription factors that are thought to act on the pre-existing $Na^+$ transport machinery [9, 39]. Three hours of aldosterone treatment increase the maximal activity of the Na–K–ATPase in mammalian cortical collecting-duct cells by increasing its cell surface expression of pre-existing $Na^+$ pumps [41]. Moreover, it has been found that aldosterone can significantly increase the phosphorylation of ENaC β and γ subunits during the early response period (3 h) without affecting their synthesis suggesting that phosphorylation could mediate the activation of pre-existing channels [35].

Finally, it has been found that the phosphorylation states of both NCC and SPAK, but not their expression levels, are increased by low-salt diets and by the administration of exogenous aldosterone in mouse DCTs [10]. These studies suggest the SPAK–NCC cascade as a target of aldosterone action in the kidney in which both the $Na^+$ transporter and the involved kinase are up-regulated in a post-translation phase.

In agreement with these findings, we suggest that SPAK–NKCC2 is a novel effector system of aldosterone in hypertensive MHS rats, which, by stimulating the phosphorylation but not the synthesis of both NKCC2 and SPAK kinase, may account for the maintenance of the hypertensive state in this strain of rats.

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## References

1. Ackermann D, Gresko N, Carrel M, Loffing-Cueni D, Habermehl D, Gomez-Sanchez C, Rossier BC, Loffing J (2010) In vivo nuclear translocation of mineralocorticoid and glucocorticoid receptors in rat kidney: differential effect of corticosteroids along the distal tubule. Am J Physiol Ren Physiol 299:F1473–F1485

2. Alvarez-Guerra M, Garay RP (2002) Renal Na-K-Cl cotransporter NKCC2 in Dahl salt-sensitive rats. J Hypertens 20:721–727
3. Bianchi G, Baer PG, Fox U, Duzzi L, Pagetti D, Giovannetti AM (1975) Changes in renin, water balance, and sodium balance during development of high blood pressure in genetically hypertensive rats. Circ Res 36:153–161
4. Bianchi G, Fox U, Di Francesco GF, Giovanetti AM, Pagetti D (1974) Blood pressure changes produced by kidney cross-transplantation between spontaneously hypertensive rats and normotensive rats. Clin Sci Mol Med 47:435–448
5. Capasso G, Rizzo M, Evangelista C, Ferrari P, Geelen G, Lang F, Bianchi G (2005) Altered expression of renal apical plasma membrane Na+ transporters in the early phase of genetic hypertension. Am J Physiol Ren Physiol 288:F1173–F1182
6. Capasso G, Rizzo M, Garavaglia ML, Trepiccione F, Zacchia M, Mugione A, Ferrari P, Paulmichl M, Lang F, Loffing J, Carrel M, Damiano S, Wagner CA, Bianchi G, Meyer G (2008) Upregulation of apical sodium-chloride cotransporter and basolateral chloride channels is responsible for the maintenance of salt-sensitive hypertension. Am J Physiol Ren Physiol 295:F556–F567
7. Carmosino M, Gimenez I, Caplan M, Forbush B (2008) Exon loss accounts for differential sorting of Na-K-Cl cotransporters in polarized epithelial cells. Mol Biol Cell 19:4341–4351
8. Carmosino M, Rizzo F, Procino G, Basco D, Valenti G, Forbush B, Schaeren-Wiemers NN, Caplan MJ, Svelto M (2010) MAL/VIP17, a new player in the regulation of NKCC2 in the kidney. Mol Biol Cell 21:3985–3997
9. Chen SY, Bhargava A, Mastroberardino L, Meijer OC, Wang J, Buse P, Firestone GL, Verrey F, Pearce D (1999) Epithelial sodium channel regulated by aldosterone-induced protein sgk. Proc Natl Acad Sci USA 96:2514–2519
10. Chiga M, Rai T, Yang SS, Ohta A, Takizawa T, Sasaki S, Uchida S (2008) Dietary salt regulates the phosphorylation of OSR1/SPAK kinases and the sodium chloride cotransporter through aldosterone. Kidney Int 74:1403–1409
11. Delpire E, Gagnon KB (2008) SPAK and OSR1: STE20 kinases involved in the regulation of ion homoeostasis and volume control in mammalian cells. Biochem J 409:321–331
12. Dowd BF, Forbush B (2003) PASK (proline-alanine-rich STE20-related kinase), a regulatory kinase of the Na-K-Cl cotransporter (NKCC1). J Biol Chem 278:27347–27353
13. Efendiev R, Krmar RT, Ogimoto G, Zwiller J, Tripodi G, Katz AI, Bianchi G, Pedemonte CH, Bertorello AM (2004) Hypertension-linked mutation in the adducin alpha-subunit leads to higher AP2-mu2 phosphorylation and impaired Na+, K+−ATPase trafficking in response to GPCR signals and intracellular sodium. Circ Res 95:1100–1108
14. Escoubet B, Coureau C, Blot-Chabaud M, Bonvalet JP, Farman N (1996) Corticosteroid receptor mRNA expression is unaffected by corticosteroids in rat kidney, heart, and colon. Am J Physiol 270: C1343–C1353
15. Ferrandi M, Salardi S, Parenti P, Ferrari P, Bianchi G, Braw R, Karlish SJ (1990) Na+/K+/Cl(−)-cotransporter mediated Rb+ fluxes in membrane vesicles from kidneys of normotensive and hypertensive rats. Biochim Biophys Acta 1021:13–20
16. Ferrandi M, Tripodi G, Salardi S, Florio M, Modica R, Barassi P, Parenti P, Shainskaya A, Karlish S, Bianchi G, Ferrari P (1996) Renal Na, K-ATPase in genetic hypertension. Hypertension 28:1018–1025
17. Ferrari P, Ferrandi M, Minotti E, Duzzi L, Bianchi G (1993) Effect of canrenone and hydrochlorothiazide on the development of hypertension in rat models of genetic hypertension. J Hypertens Suppl 11(5):S330–S331
18. Flatman PW (2008) Cotransporters, WNKs and hypertension: an update. Curr Opin Nephrol Hypertens 17:186–192
19. Flemmer AW, Gimenez I, Dowd BF, Darman RB, Forbush B (2002) Activation of the Na-K-Cl cotransporter NKCC1 detected with a phospho-specific antibody. J Biol Chem 277:37551–37558
20. Geng Y, Hoke A, Delpire E (2009) The Ste20 kinases Ste20-related proline-alanine-rich kinase and oxidative-stress response 1 regulate NKCC1 function in sensory neurons. J Biol Chem 284:14020–14028
21. Grisk O, Rettig R (2001) Renal transplantation studies in genetic hypertension. News Physiol Sci 16:262–265
22. Hughes CA, Bennett V (1995) Adducin: a physical model with implications for function in assembly of spectrin-actin complexes. J Biol Chem 270:18990–18996
23. Kim S, Hamaguchi A, Shinkawa T, Kato Y, Tsuchiya N, Miura K, Ohta K, Iwao H (1996) Molecular effects of M17055, furosemide and thiazide on cardiac hypertrophy of spontaneously hypertensive rats. JPET 279(2):983–990
24. Lloyd-MacGilp SA, Torielli L, Bechtel S, Tripodi G, Gomez-Sanchez CE, Zagato L, Bernhardt R, Kenyon CJ (2002) Mutations in aldosterone synthase gene of Milan hypertensive rats: phenotypic consequences. Am J Physiol Endocrinol Metab 282:E608–E617
25. Manunta P, Cusi D, Barlassina C, Righetti M, Lanzani C, D'Amico M, Buzzi L, Citterio L, Stella P, Rivera R, Bianchi G (1998) Alpha-adducin polymorphisms and renal sodium handling in essential hypertensive patients. Kidney Int 53:1471–1478
26. Melzi ML, Syren ML, Assael BM, Sereni F, Aperia A (1991) Increased renal tubular Na-K-ATPase activity in Milan hypertensive rats in the prehypertensive period. Pediatr Nephrol 5:700–703
27. Meneton P, Jeunemaitre X, de Wardener HE, MacGregor GA (2005) Links between dietary salt intake, renal salt handling, blood pressure, and cardiovascular diseases. Physiol Rev 85:679–715
28. Moriguchi T, Urushiyama S, Hisamoto N, Iemura S, Uchida S, Natsume T, Matsumoto K, Shibuya H (2005) WNK1 regulates phosphorylation of cation-chloride-coupled cotransporters via the STE20-related kinases, SPAK and OSR1. J Biol Chem 280:42685–42693
29. Oberleithner H, Vogel U, Kersting U, Steigner W (1990) Madin-Darby canine kidney cells. II. Aldosterone stimulates Na+/H+and Cl-/HCO3- exchange. Pflugers Arch 416:533–539
30. Piechotta K, Lu J, Delpire E (2002) Cation chloride cotransporters interact with the stress-related kinases Ste20-related proline-alanine-rich kinase (SPAK) and oxidative stress response 1 (OSR1). J Biol Chem 277:50812–50819
31. Ponce-Coria J, San-Cristobal P, Kahle KT, Vazquez N, Pacheco-Alvarez D, de Los HP, Juarez P, Munoz E, Michel G, Bobadilla NA, Gimenez I, Lifton RP, Hebert SC, Gamba G (2008) Regulation of NKCC2 by a chloride-sensing mechanism involving the WNK3 and SPAK kinases. Proc Natl Acad Sci USA 105:8458–8463
32. Rafiqi FH, Zuber AM, Glover M, Richardson C, Fleming S, Jovanovic S, Jovanovic A, O'Shaughnessy KM, Alessi DR (2010) Role of the WNK-activated SPAK kinase in regulating blood pressure. EMBO Mol Med 2:63–75
33. Richardson C, Alessi DR (2008) The regulation of salt transport and blood pressure by the WNK-SPAK/OSR1 signalling pathway. J Cell Sci 121:3293–3304
34. Schwartz MJ, Kokko JP (1980) Urinary concentrating defect of adrenal insufficiency. Permissive role of adrenal steroids on the hydroosmotic response across the rabbit cortical collecting tubule. J Clin Invest 66:234–242
35. Shimkets RA, Lifton R, Canessa CM (1998) In vivo phosphorylation of the epithelial sodium channel. Proc Natl Acad Sci USA 95:3301–3305
36. Simon DB, Karet FE, Hamdan JM, DiPietro A, Sanjad SA, Lifton RP (1996) Bartter's syndrome, hypokalaemic alkalosis with hypercalciuria, is caused by mutations in the Na-K-2Cl cotransporter NKCC2. Nat Genet 13:183–188

37. Sonalker PA, Tofovic SP, Jackson EK (2004) Increased expression of the sodium transporter BSC-1 in spontaneously hypertensive rats. J Pharmacol Exp Ther 311:1052–1061
38. Sonalker PA, Tofovic SP, Jackson EK (2007) Cellular distribution of the renal bumetanide-sensitive Na-K-2Cl cotransporter BSC-1 in the inner stripe of the outer medulla during the development of hypertension in the spontaneously hypertensive rat. Clin Exp Pharmacol Physiol 34:1307–1312
39. Spindler B, Verrey F (1999) Aldosterone action: induction of p21 (ras) and fra-2 and transcription-independent decrease in myc, jun, and fos. Am J Physiol 276:C1154–C1161
40. Stanton BA (1986) Regulation by adrenal corticosteroids of sodium and potassium transport in loop of Henle and distal tubule of rat kidney. J Clin Invest 78:1612–1620
41. Summa V, Mordasini D, Roger F, Bens M, Martin PY, Vandewalle A, Verrey F, Feraille E (2001) Short term effect of aldosterone on Na, K-ATPase cell surface expression in kidney collecting duct cells. J Biol Chem 276:47087–47093
42. Torielli L, Tivodar S, Montella RC, Iacone R, Padoani G, Tarsini P, Russo O, Sarnataro D, Strazzullo P, Ferrari P, Bianchi G, Zurzolo C (2008) Alpha-adducin mutations increase Na/K pump activity in renal cells by affecting constitutive endocytosis: implications for tubular Na reabsorption. Am J Physiol Ren Physiol 295:F478–F487
43. Tripodi G, Valtorta F, Torielli L, Chieregatti E, Salardi S, Trusolino L, Menegon A, Ferrari P, Marchisio PC, Bianchi G (1996) Hypertension-associated point mutations in the adducin alpha and beta subunits affect actin cytoskeleton and ion transport. J Clin Invest 97:2815–2822
44. van der Lubbe N, Lim CH, Fenton RA, Meima ME, Jan Danser AH, Zietse R, Hoorn EJ (2011) Angiotensin II induces phosphorylation of the thiazide-sensitive sodium chloride cotransporter independent of aldosterone. Kidney Int 79:66–76
45. Work J, Jamison RL (1987) Effect of adrenalectomy on transport in the rat medullary thick ascending limb. J Clin Invest 80:1160–1164
46. World Health Organization (2002) The world health report 2002: reducing risks, promoting healthy life. World Health Organization-Geneva, Switzerland
47. Yang SS, Lo YF, Wu CC, Lin SW, Yeh CJ, Chu P, Sytwu HK, Uchida S, Sasaki S, Lin SH (2010) SPAK-knockout mice manifest Gitelman syndrome and impaired vasoconstriction. J Am Soc Nephrol 21:1868–1877