Hyperphosphorylation of Na-K-2Cl Cotransporter in Thick Ascending Limbs of Dahl Salt-Sensitive Rats

Gustavo R. Ares, Mohammed Z. Haque, Eric Delpire, Pablo A. Ortiz

Abstract—Salt-sensitive hypertension involves a renal defect preventing the kidney from eliminating excess NaCl. The thick ascending limb of Henle loop reabsorbs ≈30% of filtered NaCl via the apical Na-K-2Cl cotransporter (NKCC2). Higher NKCC2 activity and Cl reabsorption have been reported in the thick ascending limbs from Dahl salt-sensitive rats (DSS) fed normal salt. NKCC2 activity is primarily regulated by protein trafficking and phosphorylation at Thr^{96}/Thr^{101} via STE20- and SPS1-related proline and alanine-rich kinases and oxidative stress-responsive kinase 1. However, the mechanism for enhanced NKCC2 activity in DSS is unclear. We hypothesized that DSS exhibit enhanced NKCC2 trafficking and higher NKCC2 phosphorylation compared with Dahl salt-resistant rats on normal salt diet. We measured steady state surface NKCC2 expression and phosphorylation at Thr^{96} and Thr^{101} by surface biotinylation and Western blot. In DSS, the surface:total NKCC2 ratio was enhanced by 25% compared with Dahl salt-resistant rats ($P<0.05$) despite lower NKCC2 expression. Total NKCC2 phosphorylation at Thr^{96} and Thr^{101} was enhanced ≈5-fold in DSS thick ascending limbs. Moreover, total STE20- and SPS1-related proline and alanine-rich kinases expression, kidney-specific STE20- and SPS1-related proline and alanine-rich kinases, and oxidative stress-responsive kinase 1 were not different between strains, although STE20- and SPS1-related proline and alanine-rich kinases/oxidative stress-responsive kinase 1 phosphorylation was enhanced by 60% ($P<0.05$) in DSS rats, suggesting increased activity. We concluded that phosphorylation of NKCC2 Thr^{96} and Thr^{101} and surface:total NKCC2 ratio are enhanced in DSS rats. These differences in NKCC2 may be, in part, responsible for higher NKCC2 activity and abnormally enhanced thick ascending limb NaCl reabsorption in DSS rats. **(*Hypertension*. 2012;60:1464-1470.)**

Key Words: hypertension ■ phosphorylation ■ sodium transport ■ kinases ■ kidney ■ nephron

In ≈20% of the population, high-salt consumption leads to the development of hypertension (referred to as salt sensitivity).[1] Salt-sensitive hypertension is associated with a renal defect that prevents the kidney from eliminating excess NaCl[2] and Dahl salt-sensitive (DSS) rats exhibit a similar renal defect. The thick ascending limb (TAL) reabsorbs ≈30% of the total filtered NaCl via the apical Na-K-2Cl cotransporter (NKCC2).[3,4] In DSS rats, abnormally enhanced TAL-dependent NaCl reabsorption has been reported, but not in the proximal or distal tubule.[5] In vivo micropuncture experiments in DSS rats showed increased Cl^- reabsorption along the loop of Henle on a normal salt diet[5–8] before development of hypertension, suggesting that enhanced TAL-dependent NaCl reabsorption contributes to hypertension. However, little is known regarding the transporters and molecular mechanisms that mediate enhanced TAL NaCl reabsorption in DSS rats.

Isolated, perfused TALs from DSS rats exhibit higher Cl^- reabsorption and lumen-positive potential.[8–10] Alvarez-Guerra and Garay[11] showed that DSS rats fed a normal salt diet exhibit a higher natriuretic response to the loop diuretic bumetanide compared with Dahl salt-resistant (DSR) rats. They also found that NKCC2 activity and intracellular Cl^- were higher in TALs from DSS rats. These data suggest that enhanced NKCC2 activity is likely responsible for abnormally enhanced NaCl reabsorption along the loop of Henle in DSS rats. However, the molecular mechanisms responsible for the enhanced NKCC2 activity in DSS rats are not fully understood.

Approximately 5% of the total pool of NKCC2 is located in the apical membrane of rat TALs.[12–15] We showed that trafficking of NKCC2 into and out of the apical membrane (endocytosis, exocytosis, and recycling) is responsible for maintaining surface NKCC2 at steady state levels.[12–16] Moreover, we and others have reported that the capacity of the TAL to reabsorb NaCl is directly related to the amount of NKCC2 at the surface. For example, vasopressin and cAMP stimulate NaCl reabsorption by increasing surface NKCC2 levels.[14,15,17] In contrast, cGMP, the second messenger for nitric oxide and natriuretic peptides, inhibits NaCl reabsorption by decreasing surface NKCC2 levels in the TAL.[12] Recently, we found that a high-salt diet enhanced surface NKCC2 and TAL transport in DSS rats.[16] However, it is not known whether surface NKCC2 levels are different between DSS

Received July 11, 2012; first decision July 30, 2012; revision accepted September 17, 2012.

From the Hypertension and Vascular Research Division, Department of Internal Medicine, Henry Ford Hospital, Detroit, MI (G.R.A., M.Z.H., P.A.O.); Department of Anesthesiology, Vanderbilt University School of Medicine, Nashville, TN (E.D.); Department of Physiology, Wayne State University, Detroit, MI (P.A.O.).

Correspondence to Pablo A. Ortiz, Hypertension and Vascular Research Division, Department of Internal Medicine, Henry Ford Hospital, 2799 West Grand Blvd, Detroit, MI 48202. E-mail portiz1@hfhs.org

and DSR rats fed a normal salt diet before the development of hypertension.

In addition to protein trafficking, phosphorylation of NKCC2 regulates its activity. Deletion of the phosphorylation sites Thr^{96} and Thr^{101} from the NH_2 terminus of NKCC2 eliminates NKCC2 activity in heterologous expression systems.[18] In the TAL, NKCC2 phosphorylation at Thr^{96} and Thr^{101} is detectable under baseline conditions in the absence of stimuli.[16,17] The kinases involved in phosphorylation at Thr^{96} and Thr^{101} are members of the STE20 subfamily, STE20- and SPS1-related proline and alanine-rich kinases (SPAK) and oxidative stress-responsive kinase 1 (OSR1),[19] sharing ≈67% homology.[20] In some instances, SPAK is autophosphorylated,[21] although the mechanism is not fully understood. This pathway is relevant to human hypertension because impaired regulation by upstream kinases With-No-Lysine Kinase (WNK)1[22,23] and WNK4[24,25] is responsible for some rare forms of monogenic hypertension.[26] However, it is not known whether phosphorylation of NKCC2 and expression of SPAK and OSR1 kinases are affected in DSS rats.

We previously found that surface NKCC2 was enhanced in DSS rats but decreased in DSR rats, suggesting an impaired response to high-salt diet that alters NKCC2 trafficking in DSS rats. Phosphorylation of NKCC2 was not affected by high salt in either strain although it was apparent that the amount of phosphorylated NKCC2 was higher in DSS rats. Because NKCC2 activity and Cl^- reabsorption are enhanced in TALs from DSS rats fed a normal salt diet, we hypothesized that baseline NKCC2 phosphorylation and surface NKCC2 expression are different in TALs from DSS and DSR rats.

Methods

Suspensions of Medullary TALs

Male DSS and DSR rats (8–9 weeks old) weighing 200 to 250 g (Harlan, Indianapolis, IN) were maintained on a diet containing 0.22% sodium (which provides excess Na^+[27] and has been widely used in tubule perfusion experiments to stabilize baseline Na^+ transport) and 1.1% potassium (Purina, Richmond, IN) with water ad libitum for ≥14 days after arrival to our facility. All animal protocols were approved by the Henry Ford Hospital Institutional Animal Care Committee. Suspensions of medullary TALs were obtained as described previously.[12–16]

Measurement of Steady State Surface NKCC2 Levels

To measure steady state surface NKCC2 levels, TAL suspensions were cooled to 4°C and surface proteins labeled with NHS-SS-biotin as we previously described.[12–14,16] Optical densities from surface and intracellular NKCC2 bands were used to calculate the ratio of surface:total NKCC2. All optical densities were within the linear range of the scanner. In all experiments, we used 75 to 100 μg of initial protein lysate to measure steady state surface NKCC2 levels within a linear range. Control experiments showed that the intracellular protein GAPDH was absent in the biotinylated surface fraction but abundant in the intracellular fraction, indicating that the biotinylated fraction contained only surface proteins.

Western Blot

Protein expression was performed as we previously described.[12–14,16] Primary antibodies were incubated for 120 minutes at room temperature in blocking buffer (containing 2% BSA) at a dilution of 1/20 000 for rabbit antirat NKCC2 (directed to amino acids 859–873 in the rat NKCC2 COOH terminus), and 1/5000 for the chicken antirat NKCC2 NH_2 terminus (L320 epitope). For phospho-NKCC2 at Thr^{96} and Thr^{101}, we used the rabbit polyclonal R5 antibody,[17,28] kindly provided by Dr Biff Forbush (Yale University, New Haven, CT). Total SPAK and OSR1 were detected with polyclonal antibodies from Cell Signaling Technology (Danvers, MA).[29,30] Phospho-SPAK/OSR1 was detected with a previously characterized sheep antibody that recognizes both SPAK and OSR1 from the College of Life Sciences, University of Dundee (Dundee, Scotland, United Kingdom).[31] Phospho-SPAK/OSR1 was also examined with a previously characterized rabbit antiphospho-SPAK/OSR1 kindly provided by Dr Shibuya.[23] Kidney-specific SPAK (KS-SPAK) and total SPAK were detected with a previously characterized rabbit anti-COOH terminal SPAK antibody.[20,32] Exposure times and amounts of protein loaded were optimized so that optical densities were linear.

Statistics

Data are expressed as mean±SEM. One-way ANOVA was used to determine statistical differences between means in different treatment groups when measuring surface and total NKCC2, SPAK, OSR1, and phospho-SPAK/OSR1 by Western blot and fluorescence imaging. Differences between means were considered significant at $P<0.05$.

Results

Surface:Total NKCC2 Ratio Is Higher in DSS Rats

NKCC2 activity[11] and net Cl^- reabsorption are reportedly enhanced in DSS rats fed a normal salt diet.[5–8] Under a normal salt diet (0.22% Na^+), baseline systolic blood pressure measured by tail cuff was slightly higher in DSS compared with DSR rats (132±3 versus 116±3 mm Hg; $P<0.05$). We hypothesized that NKCC2 trafficking is enhanced in DSS compared with DSR rats. To test our hypothesis, we measured surface and total NKCC2 levels in TALs from DSS and DSR rats fed normal salt (0.22% Na^+). TALs from DSS rats were compared with TALs isolated from DSR rats on the same membranes. Opposite to our hypothesis, surface NKCC2 levels in DSS rats were 27±6% lower (DSR: 14±2 versus DSS: 10±1 optical density; n=7; $P<0.05$; Figure 1A). Surprisingly, total NKCC2 expression normalized to GAPDH was 42±6% lower in DSS rats (DSR: 29±5 versus DSS: 17±4 optical density; n=7; $P<0.05$; Figure 1B). Because total NKCC2 expression was much lower at baseline in the DSS rats, the best estimate of NKCC2 trafficking to the apical membrane is the surface:total ratio, which indicates the fraction of the total pool of NKCC2 located in the plasma membrane. We found a 25±9% (n=7; $P<0.05$) increase in the surface:total NKCC2 ratio in DSS rats TALs (DSR, 5.1±0.7%; DSS, 6.3±0.6%; Figure 1C and 1D). These data indicate that despite having lower total NKCC2 expression, the fraction of NKCC2 located at the surface is higher in DSS rats. Nevertheless, the net amount of NKCC2 at the surface is 25% lower in DSS rats, suggesting that an additional mechanism mediates enhanced NKCC2 activity.

NKCC2 Phosphorylation Is Enhanced in DSS Rats

Phosphorylation of Thr^{96} and Thr^{101} is required for NKCC2 activity.[17,18] Therefore, we studied whether NKCC2 phosphorylation at Thr^{96} and Thr^{101} is enhanced in DSS compared with DSR rats. For this, we used an antibody that recognizes phosphorylated Thr^{96} and Thr^{101} and normalized this

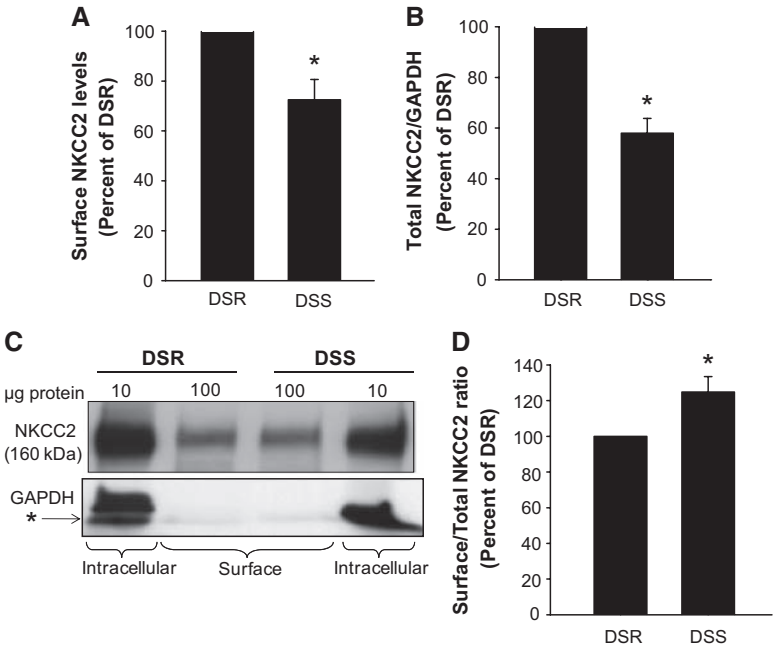

Figure 1. Surface and total Na-K-2Cl cotransporter (NKCC2) expression in the thick ascending limbs (TALs) from Dahl salt-resistant (DSR) and Dahl salt-sensitive (DSS) rats on a normal salt diet. **A**, Cumulative optical density data showing surface NKCC2 levels in TALs from DSR and DSS rats (n=7; $P<0.05$). Data were normalized to control DSR TALs, which was set to 100. **B**, Cumulative optical density data showing total NKCC2 expression normalized to GAPDH expression in TALs from DSR and DSS rats (n=7; $P<0.05$). Data were normalized to control DSR TALs, which was set to 100. **C**, Representative Western blot of surface NKCC2 (from 100 µg of protein) and intracellular NKCC2 (from 10 µg of protein). Surface and intracellular samples from DSR and DSS were loaded in the same gel for comparison. GAPDH was not detected in the surface fraction. Asterisk (*) at the bottom of the panel emphasizes the detection of degradation products present in most lanes. Note that GAPDH runs above those nonspecific products and is absent in the lanes from the surface fraction. **D**, Cumulative data comparing the surface:total NKCC2 ratio in DSR and DSS rats on a normal salt diet. The bar graph (derived from individual experiments shown in Figure 1A and 1B) shows surface:total ratios normalized to DSR, which was set to 100 (n=7; $P<0.05$).

signal to total NKCC2 expression.[16,17,28] We found that Thr[96] and Thr[101] phosphorylation corrected over total NKCC2 levels was ≈5-fold higher in TALs from DSS rats maintained on normal salt diet (DSR, 100±25%; DSS, 476±105%; n=6; $P<0.05$; Figure 2A and 2B). Next, we measured the surface:total ratio of phosphorylated NKCC2 in DSS rats TALs and compared with to DSR rats. As expected the signal for phosphorylated NKCC2 in the surface and intracellular fractions was higher in DSS rats. However, the fraction of phosphorylated NKCC2 at the surface was not significantly different between strains (DSR, 8.7±0.7%; DSS, 7.0±1.0%; n=4; Figure 2C). Overall, NKCC2 phosphorylation at Thr[96] and Thr[101] in the surface and intracellular compartments is higher in DSS compared with the DSR rats despite paradoxically decreased total NKCC2 expression levels.

Finally, we compared phosphorylated NKCC2 between DSS rats and the salt resistant Sprague Dawley strain. When analyzed in the same gels, phosphorylated NKCC2 corrected to total NKCC2 was 91% higher in DSS rats (Sprague Dawley, 100±8%; DSS, 191±34%; n=5; $P<0.05$; data not shown). These data suggest that enhanced phosphorylation of NKCC2 is maintained in DSS rats when compared with genetically distant salt-resistant strains and may be involved in salt sensitivity.

SPAK/OSR1 Phosphorylation Is Increased in DSS Rats

To date the only 2 kinases known to phosphorylate NKCC2 at Thr[96] and Thr[101] are SPAKs and OSR1 kinases.[19,20,33] Therefore, we studied whether expression of total and phosphorylated SPAK/OSR1 was different between DSR and DSS rats fed a normal salt diet. Total SPAK, normalized to GAPDH was not different between DSR and DSS (DSR, 100±10.6%; DSS, 84.1±9.4%; n=6; P value not significant; Figure 3A and 3B). We also measured the expression of a newly described KS-SPAK variant. KS-SPAK exerts an inhibitory effect on NKCC2 phosphorylation in heterologous expression systems,[32] and a decrease in KS-SPAK expression could lead to hyperphosphorylation of NKCC2 in DSS rats. Expression of KS-SPAK was not different between strains (Figure 3C).

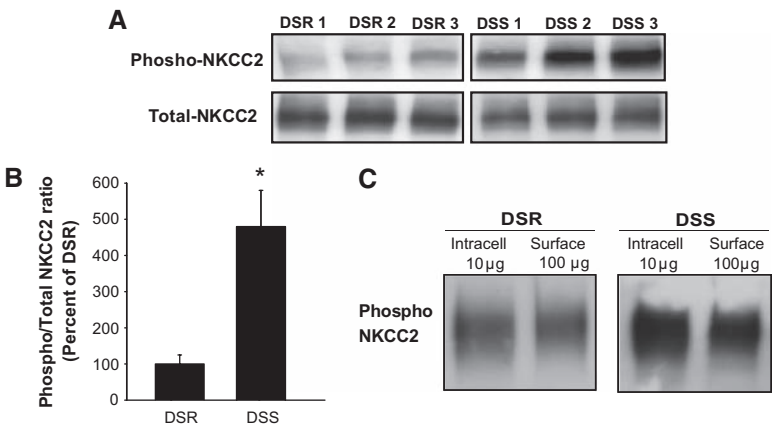

Figure 2. Surface and total phosphorylated Na-K-2Cl cotransporter (NKCC2) in the thick ascending limbs (TALs) from Dahl salt-resistant (DSR) and Dahl salt-sensitive (DSS) rats fed a normal salt diet. **A**, Representative Western blot from 3 DSR and 3 DSS rats showing the phosphorylation of NKCC2 at Thre[96,101](phospho-NKCC2) and total NKCC2 expression in whole TAL lysates. Each lane represents the signal for 5 µg of total loaded protein. **B**, Cumulative data showing phospho-NKCC2 normalized to total NKCC2. Data were normalized to DSR rats, set to 100 (n=8 DSR and n=6 DSS; $P<0.05$). **C**, Representative Western blot showing phosphorylated NKCC2 in the surface (from 100 µg of protein) and intracellular fractions (from 10 µg of protein) obtained from DSR and DSS TALs that were surface biotinylated and blotted for phosphorylated Thre[96,101]. Most phosphorylated NKCC2 is found in the intracellular fraction, and optical densities are greater in DSS.

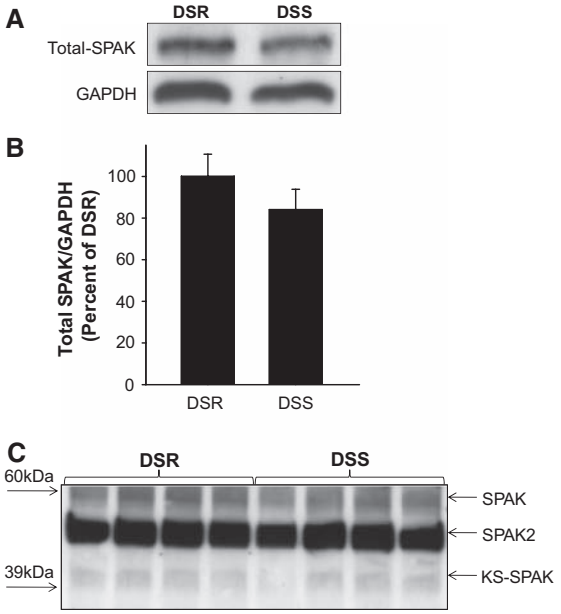

Figure 3. Expression of total STE20- and SPS1-related proline and alanine-rich kinases (SPAK) and SPAK variants in the thick ascending limbs (TALs) from Dahl salt-resistant (DSR) and Dahl salt-sensitive (DSS) rats on a normal salt diet. **A**, Representative Western blot showing SPAK (full length) and GAPDH expression in whole TAL lysates from DSR and DSS rats. Each lane represents the signal for 5 μg of total loaded protein. **B**, Cumulative data showing total SPAK corrected by GAPDH in DSR and DSS rats (n=6). Data were normalized to DSR which was set to 100. **C**, Representative Western blot showing SPAK variants detected in whole TAL lysates with an anti-COOH terminal SPAK antibody. Full length (60 kDa), SPAK2 variant (50–52 kDa), and kidney-specific SPAK (KS-SPAK; 40 kDa) are detected in TALs form DSR and DSS rats (from 10 μg of total loaded protein).

Total OSR1 expression, normalized to GAPDH, was also not different between groups (DSR, 100.1±8.3%; DSS, 109.2±10.4%; n=9; *P* value not significant; Figure 4). SPAK and OSR1 are autophosphorylated when they are activated.[21] Thus, we measured the phosphorylation of SPAK/OSR1. Given that antibodies recognize a conserved phosphorylation site in SPAK and OSR1 and the kinases differ by 2 kDa, we could not accurately distinguish between them. Thus we measured the 58-to 60-kDa band recognized by phospho-antibodies for the full-length proteins. We found that SPAK/OSR1 phosphorylation was higher in TALs from DSS rats (DSR, 100%; DSS, 157±14%; n=6; $P<0.05$; Figure 5). These data suggest that SPAK/OSR1 phosphorylation is slightly enhanced in DSS rats. Taken together, these data suggest that enhanced NKCC2 phosphorylation in DSS rats on a normal salt diet may be, in part, because of enhanced SPAK or OSR1 activity.

Discussion

NaCl reabsorption and NKCC2 activity are enhanced in TALs from DSS rats fed a normal salt diet whereas other nephron segments showed unchanged or even decreased NaCl reabsorption.[34–36] NaCl reabsorption in the TAL is mediated by NKCC2.[37,38] We hypothesized that during a normal salt diet, DSS rats exhibit higher surface NKCC2 levels and higher NKCC2 phosphorylation compared with DSR rats. We found

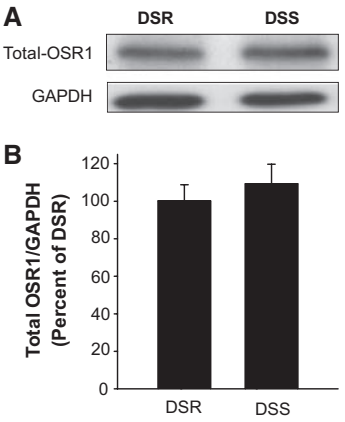

Figure 4. Expression of total oxidative stress-responsive kinase 1 (OSR1) in the thick ascending limbs (TALs) from Dahl salt-resistant rats (DSR) and Dahl salt-sensitive rats (DSS) rats on a normal salt diet. **A**, Representative Western blot showing OSR1 expression in TALs from DSR and DSS rats. Each lane represents the signal for 5 μg total loaded protein. A Band detected at 58 to 60 kDa was selected for measurement. **B**, Cumulative data showing total OSR1 corrected by GAPDH in TALs from DSR and DSS rats (n=8). Data were normalized to DSR which was set to 100.

that NKCC2 expression was lower in TALs from DSS rats, whereas the fraction of NKCC2 located in the surface was higher in DSS rats. Despite lower NKCC2 expression, baseline NKCC2 phosphorylation was 5-fold higher in DSS rats. There was a paradoxical decrease in total NKCC2 expression in DSS rats. We also found that phosphorylated SPAK/OSR1was higher in DSS rats, whereas total expression of SPAK and OSR1 was similar between strains. Therefore, we concluded

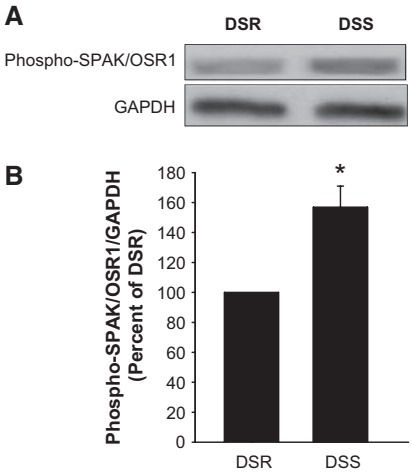

Figure 5. Expression of phosphorylated STE20- and SPS1-related proline and alanine-rich kinases (SPAK)/oxidative stress-responsive kinase 1 (OSR1) in the thick ascending limbs (TALs) from Dahl salt-resistant (DSR) and Dahl salt-sensitive (DSS) rats on a normal salt diet. **A**, Representative Western blot showing phospho-SPAK/OSR1 normalized to GAPDH in TALs from DSR and DSS rats. Each lane represents the signal for 10 μg of loaded protein. **B**, Cumulative data showing the densitometry of phospho-SPAK/OSR1 normalized to GAPDH detected with phospho-SPAK/OSR1 antibody obtained from Dr Shibuya (n=4). Data were normalized to DSR, which was set to 100. Similar results were obtained in a different set of experiments with an antibody generated at the University of Dundee.

that both trafficking and phosphorylation of NKCC2 are affected in DSS rats and that enhanced activity of SPAK or OSR1 may be involved in higher NKCC2 phosphorylation.

It is not clear why DSS rats exhibit enhanced NKCC2 activity[11] and higher baseline NaCl reabsorption compared with DSR rats.[6,7,36] Approximately 5% of total NKCC2 is present at the apical surface of the TAL, which is directly related to NaCl reabsorption.[12–16] This is supported by experiments showing that cAMP increases NaCl reabsorption by enhancing surface NKCC2 levels,[14,15] whereas cGMP inhibits NaCl reabsorption by decreasing surface NKCC2 levels.[12] Therefore, to explain the higher NKCC2 activity in DSS rats we focused our attention on the amount of surface NKCC2 in DSS rats. Contrary to our hypothesis, we found that net surface NKCC2 was 25% lower in DSS rats. Surprisingly, total NKCC2 protein expression was 45% lower in DSS rats. Because of the large decrease in total NKCC2, the fraction of total NKCC2 present at the surface is 25% higher in DSS rats on a normal salt diet. In agreement with our data, Alvarez-Guerra and Garay[11] have found an even larger decrease in total NKCC2 in DSS rats. Another group of investigators found no difference in total NKCC2 expression between DSS and DSS-BN13 consomic rats.[39] In our experiments, the decrease in total NKCC2 expression was observed with antibodies recognizing the NH_2 or COOH terminus of NKCC2. Although it is not clear to us why total NKCC2 is lower, it is possible that this occurs as part of a negative feedback to minimize NaCl reabsorption by decreasing total NKCC2 expression. It is clear from our data that total NKCC2 expression is not correlated with the transporter activity.

Phosphorylation of NKCC2 NH_2 terminus threonines (Thr^{96} and Thr^{101}) is detectable in the absence of stimuli in mouse and rat TALs,[17,28] and deleting these threonines nearly eliminates baseline NKCC2 activity in heterologous expression systems.[18,28] Thus, this mechanism may contribute to enhanced NKCC2 activity in DSS rats. Here, we found that baseline NKCC2 phosphorylation was 5-fold higher in DSS rats on a normal salt diet. In a previous study, we examined the effect of a high-salt diet (4–5 days)[16] on NKCC2 phosphorylation in DSS and DSR rats and found no differences in either strain. Taken together, our data suggest that NKCC2 phosphorylation is enhanced under baseline conditions in DSS rats and remains elevated during high-salt intake, despite higher NKCC2 surface expression. Most likely, enhanced phosphorylation and trafficking contribute to increased NaCl reabsorption in DSS rats. In addition to Thr^{96} and Thr^{101}, NKCC2 is also phosphorylated at Ser^{126} and Ser^{874} by protein kinase A during stimulation of cAMP signaling.[19,40] It is not known whether these sites are also affected in DSS rats, or whether the response to cAMP-generating hormones is altered in TALs.

Our data show that NKCC2 phosphorylation is easily detected in both the surface and intracellular compartments. The surface:total ratio of phosphorylated NKCC2 was similar between strains, and the total amount of phosphorylated NKCC2 was 5-fold higher in DSS rats. Thus our data indicate that phosphorylated NKCC2 at the surface is ≥3-fold higher in DSS rats after taking into account the lower surface NKCC2 in DSS rats (Figure 1A). The fraction of phosphorylated NKCC2 at the surface was 7% to 8%, which is higher than that obtained when measuring total NKCC2 (4–5%). These data suggest that phosphorylation is likely to alter NKCC2 trafficking. However, the precise effect of phosphorylation on NKCC2 trafficking is not fully understood.

Our study provides novel information regarding the molecular mechanisms that may be involved in higher NKCC2 activity in DSS rats. Most likely net NKCC2 activity is determined by both the number of transporters at the surface and the phosphorylated NKCC2 fraction of transporters at the plasma membrane. Despite a 25% lower surface NKCC2, the amount of phosphorylated NKCC2 at the surface is ≥3× larger in DSS rats suggesting that enhanced phosphorylation is responsible for higher activity. However, our data also show that the surface:total NKCC2 ratio was higher in DSS rats. Surface NKCC2 is maintained at a steady state by a balance between exocytosis (including recycling) and endocytosis.[12–15] A higher surface:total NKCC2 ratio in DSS rats indicates that either the rate of exocytic delivery is increased or the rate of endocytosis is decreased. Because NKCC2 is phosphorylated both in the intracellular compartment and apical surface (Figure 2C), the residence time of phosphorylated NKCC2 at the surface might affect the rate of trafficking into and out of the apical membrane, which likely determines the net activity of NKCC2. Thus, our data suggest that both phosphorylation and trafficking are likely involved in enhanced NKCC2 activity in DSS rats.

Only 2 kinases are known to phosphorylate NKCC2 at Thr^{96} and Thr^{101}, SPAKs and OSR1,[19,20] both of which are found in TAL lysates. Three SPAK variants, full-length, SPAK2, and a shorter KS-SPAK, were detected in TAL lysates. OSR1 (sharing 67% homology with SPAK) phosphorylates NKCC2[20,29] and was also detected in TAL lysates. The KS splice variant of SPAK has only been reported in the mouse kidney. KS-SPAK inhibits NKCC2 phosphorylation by full-length SPAK in heterologous expression system (*Xenopus laevis* oocytes).[32] Using C-terminus directed antibodies that recognize all SPAK variants, we observed 3 bands, the first band at 61 kDa (corresponding with the predicted mass of full-length SPAK); a second predominant band at 50 to 52 kDa, named SPAK2; and a weaker band at 40 kDa reportedly corresponding with KS-SPAK.[32] We found no significant differences in band densities in any of these isoforms between DSS and DSR rats. The relative amounts of SPAK variants are different from those reported in mouse whole-kidney homogenates. In rat TAL lysates the 50-kDa SPAK isoform was predominant (≈25-fold higher expression). Total OSR1, detected at 58 kDa, was not different between strains. To examine whether enhanced NKCC2 phosphorylation may be correlated with higher activity of SPAK or OSR1 we monitored their phosphorylation at Thr^{373}, which is required for SPAK and OSR1 activity.[19,20,33,41] The antibodies used[19,23] do not differentiate between SPAK and OSR1 given that the activation phospho-site is conserved in these 2 kinases. Because the mass of SPAK and OSR1 is similar (60 and 58 kDa, respectively) we think these phospho-antibodies cannot accurately differentiate between them. We found a small but significant increase in SPAK/OSR1 phosphorylation in DSS rats. Because both SPAK and OSR1 are able to phosphorylate NKCC2 threonines 96 and 101, it is possible that their enhanced activity is involved in higher NKCC2 phosphorylation in DSS rats.

The regulation of NKCC2 phosphorylation by SPAK and OSR1 is likely complex and may involve additional kinases and other protein–protein interactions. The mechanisms by which SPAK and OSR1 are activated are not fully understood. In the distal convoluted tubule the upstream kinases WNK1 and WNK4 regulate SPAK and OSR1 phosphorylation, which in turn modulate Na-Cl co-transporter Decreased WNK1 activity or expression, or enhanced WNK4 activity stimulate Na-Cl co-transporter phosphorylation via SPAK/OSR1, which is correlated with hypertension in humans.[24,25,42] The relative expression of these kinases and their kidney-specific splice variants in the TAL is unclear. However, if they were expressed in TALs and regulated in a similar manner as in the distal convoluted tubule, it could be hypothesized that defects in WNK1 and 4 could enhance NKCC2 phosphorylation in DSS rats via SPAK/OSR1. Little is known about which autacoids and hormones stimulate the SPAK and OSR1 pathway or NKCC2 phosphorylation. Vasopressin is known to enhance NKCC2 phosphorylation at Thr^{96} and Thr^{101} in mouse TALs.[17] However, under a normal salt diet and fluid intake as in this study, vasopressin levels are not likely elevated in DSS or DSR rats. Given the genetic basis of salt-sensitive hypertension, it is likely that proteins involved in NKCC2 phosphorylation are differentially regulated or expressed in TALs of DSS rats, altering NKCC2 activity independently of autacoids and hormones. In addition, regulation of TAL transport by autacoids such as nitric oxide and 20-HETE is known to be impaired in DSS rats.[9,10,43,44] It is not known whether SPAK, OSR1, and NKCC2 phosphorylation is regulated by these endogenous autacoids. Our previous data[16] showed that enhanced NKCC2 phosphorylation and activity are maintained during a high-salt diet. In addition, enhanced NKCC2 activity was maintained in high-salt-fed DSS rats in which blood pressure was normalized with a distal tubule diuretic dissociating NKCC2 activity from blood pressure. Thus, our data and those of others indicate that an intrinsic genetic defect in DSS leads to enhanced NKCC2 phosphorylation. However, it is possible that enhanced NKCC2 phosphorylation in DSS rats fed a normal salt diet is somewhat influenced by the higher baseline blood pressure observed in DSS rats (15 mm Hg). The effects of acute changes in blood pressure or chronically enhanced blood pressure on NKCC2 phosphorylation have not been studied.

To our knowledge, our study is the first to examine NKCC2 phosphorylation and surface NKCC2 levels in DSS and DSR rats on a normal salt diet. Our data indicate that phosphorylation and surface:total NKCC2 ratio are increased in DSS rats maintained on a normal salt diet and this may, in part, contribute to enhanced NKCC2-dependent NaCl reabsorption by the TAL.

Perspectives

Salt-sensitive hypertension is a complex polygenic condition affecting several organs. Enhanced renal tubular NaCl reabsorption is likely involved in the initial rise in blood pressure and maintenance of hypertension observed in DSS rats. Whereas enhanced NaCl reabsorption by the TAL in DSS rats has been widely reported, the molecular mechanisms by which this occurs has not been fully elucidated. Our data suggest that the intrinsic regulation of NKCC2 trafficking and phosphorylation is impaired in TALs isolated form DSS rats fed a normal salt diet, which may be related to higher NKCC2 activity in DSS rats, thereby contributing to NaCl retention and hypertension in DSS rats and human salt-sensitive patients.

Acknowledgments

We thank Dr Biff Forbush (Yale School of Medicine, New Haven, CT) for generously providing the Thr^{96}/Thr^{101} phospho-NKCC2 antibody (R5), and Dr Hiroshi Shibuya (Tokyo Medical and Dental University, Japan) for generously providing the total OSR1 and phospho-OSR1 antibody.

Sources of Funding

This work was supported, in part, by National Institutes of Health grants RO-1 HL-080409 and P01 HL090550 (sub project 2) to P.A. Ortiz. E. Delpire was supported by GM074771.

Disclosures

None.

References

1. Weinberger MH. Salt sensitivity of blood pressure in humans. *Hypertension*. 1996;27(3 pt 2):481–490.
2. Tobian L. Salt and hypertension. Lessons from animal models that relate to human hypertension. *Hypertension*. 1991;17(1 suppl):I52–I58.
3. Bennett CM, Brenner BM, Berliner RW. Micropuncture study of nephron function in the rhesus monkey. *J Clin Invest*. 1968;47:203–216.
4. Burg MB. Tubular chloride transport and the mode of action of some diuretics. *Kidney Int*. 1976;9:189–197.
5. Roman RJ, Kaldunski ML. Enhanced chloride reabsorption in the loop of Henle in Dahl salt-sensitive rats. *Hypertension*. 1991;17(6 pt 2):1018–1024.
6. Kirchner KA. Greater loop chloride uptake contributes to blunted pressure natriuresis in Dahl salt-sensitive rats. *J Am Soc Nephrol*. 1990;1:180–186.
7. Kirchner KA. Increased loop chloride uptake precedes hypertension in Dahl salt-sensitive rats. *Am J Physiol*. 1992;262(2 pt 2):R263–R268.
8. Zou AP, Drummond HA, Roman RJ. Role of 20-HETE in elevating loop chloride reabsorption in Dahl SS/Jr rats. *Hypertension*. 1996;27(3 pt 2):631–635.
9. García NH, Plato CF, Stoos BA, Garvin JL. Nitric oxide-induced inhibition of transport by thick ascending limbs from Dahl salt-sensitive rats. *Hypertension*. 1999;34:508–513.
10. Ito O, Roman RJ. Role of 20-HETE in elevating chloride transport in the thick ascending limb of Dahl SS/Jr rats. *Hypertension*. 1999;33(1 pt 2):419–423.
11. Alvarez-Guerra M, Garay RP. Renal Na-K-Cl cotransporter NKCC2 in Dahl salt-sensitive rats. *J Hypertens*. 2002;20:721–727.
12. Ares GR, Caceres P, Alvarez-Leefmans FJ, Ortiz PA. cGMP decreases surface NKCC2 levels in the thick ascending limb: role of phosphodiesterase 2 (PDE2). *Am J Physiol Renal Physiol*. 2008;295:F877–F887.
13. Ares GR, Ortiz PA. Constitutive endocytosis and recycling of NKCC2 in rat thick ascending limbs. *Am J Physiol Renal Physiol*. 2010;299:F1193–F1202.
14. Caceres PS, Ares GR, Ortiz PA. cAMP stimulates apical exocytosis of the renal Na(+)-K(+)-2Cl(-) cotransporter NKCC2 in the thick ascending limb: role of protein kinase A. *J Biol Chem*. 2009;284:24965–24971.
15. Ortiz PA. cAMP increases surface expression of NKCC2 in rat thick ascending limbs: role of VAMP. *Am J Physiol Renal Physiol*. 2006;290:F608–F616.
16. Haque MZ, Ares GR, Caceres PS, Ortiz PA. High salt differentially regulates surface NKCC2 expression in thick ascending limbs of Dahl salt-sensitive and salt-resistant rats. *Am J Physiol Renal Physiol*. 2011;300:F1096–F1104.
17. Giménez I, Forbush B. Short-term stimulation of the renal Na-K-Cl cotransporter (NKCC2) by vasopressin involves phosphorylation and membrane translocation of the protein. *J Biol Chem*. 2003;278:26946–26951.

18. Ponce-Coria J, San-Cristobal P, Kahle KT, Vazquez N, Pacheco-Alvarez D, de Los Heros P, Juárez P, Muñoz E, Michel G, Bobadilla NA, Gimenez I, Lifton RP, Hebert SC, Gamba G. Regulation of NKCC2 by a chloride-sensing mechanism involving the WNK3 and SPAK kinases. *Proc Natl Acad Sci USA*. 2008;105:8458–8463.
19. Richardson C, Sakamoto K, de los Heros P, Deak M, Campbell DG, Prescott AR, Alessi DR. Regulation of the NKCC2 ion cotransporter by SPAK-OSR1-dependent and -independent pathways. *J Cell Sci*. 2011;124(pt 5):789–800.
20. Piechotta K, Lu J, Delpire E. 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*. 2002;277:50812–50819.
21. Gagnon KB, England R, Delpire E. Characterization of SPAK and OSR1, regulatory kinases of the Na-K-2Cl cotransporter. *Mol Cell Biol*. 2006;26:689–698.
22. Vitari AC, Thastrup J, Rafiqi FH, Deak M, Morrice NA, Karlsson HK, Alessi DR. Functional interactions of the SPAK/OSR1 kinases with their upstream activator WNK1 and downstream substrate NKCC1. *Biochem J*. 2006;397:223–231.
23. Moriguchi T, Urushiyama S, Hisamoto N, Iemura S, Uchida S, Natsume T, Matsumoto K, Shibuya H. WNK1 regulates phosphorylation of cation-chloride-coupled cotransporters via the STE20-related kinases, SPAK and OSR1. *J Biol Chem*. 2005;280:42685–42693.
24. Yang CL, Zhu X, Wang Z, Subramanya AR, Ellison DH. Mechanisms of WNK1 and WNK4 interaction in the regulation of thiazide-sensitive NaCl cotransport. *J Clin Invest*. 2005;115:1379–1387.
25. Lalioti MD, Zhang J, Volkman HM, Kahle KT, Hoffmann KE, Toka HR, Nelson-Williams C, Ellison DH, Flavell R, Booth CJ, Lu Y, Geller DS, Lifton RP. Wnk4 controls blood pressure and potassium homeostasis via regulation of mass and activity of the distal convoluted tubule. *Nat Genet*. 2006;38:1124–1132.
26. Wilson FH, Disse-Nicodème S, Choate KA, et al. Human hypertension caused by mutations in WNK kinases. *Science*. 2001;293:1107–1112.
27. Martus W, Kim D, Garvin JL, Beierwaltes WH. Commercial rodent diets contain more sodium than rats need. *Am J Physiol Renal Physiol*. 2005;288:F428–F431.
28. Giménez I, Forbush B. Regulatory phosphorylation sites in the NH2 terminus of the renal Na-K-Cl cotransporter (NKCC2). *Am J Physiol Renal Physiol*. 2005;289:F1341–F1345.
29. Lin SH, Yu IS, Jiang ST, Lin SW, Chu P, Chen A, Sytwu HK, Sohara E, Uchida S, Sasaki S, Yang SS. Impaired phosphorylation of Na(+)-K(+)-2Cl(-) cotransporter by oxidative stress-responsive kinase-1 deficiency manifests hypotension and Bartter-like syndrome. *Proc Natl Acad Sci USA*. 2011;108:17538–17543.
30. Ohta A, Rai T, Yui N, Chiga M, Yang SS, Lin SH, Sohara E, Sasaki S, Uchida S. Targeted disruption of the WNK4 gene decreases phosphorylation of Na-Cl cotransporter, increases Na excretion and lowers blood pressure. *Hum Mol Genet*. 2009;18:3978–3986.
31. Filippi BM, de los Heros P, Mehellou Y, Navratilova I, Gourlay R, Deak M, Plater L, Toth R, Zeqiraj E, Alessi DR. MO25 is a master regulator of SPAK/OSR1 and MST3/MST4/YSK1 protein kinases. *EMBO J*. 2011;30:1730–1741.
32. McCormick JA, Mutig K, Nelson JH, Saritas T, Hoorn EJ, Yang CL, Rogers S, Curry J, Delpire E, Bachmann S, Ellison DH. A SPAK isoform switch modulates renal salt transport and blood pressure. *Cell Metab*. 2011;14:352–364.
33. Gagnon KB, Delpire E. On the substrate recognition and negative regulation of SPAK, a kinase modulating Na+-K+-2Cl- cotransport activity. *Am J Physiol, Cell Physiol*. 2010;299:C614–C620.
34. Hawk CT, Schafer JA. Effects of AVP and deoxycorticosterone on Na+ and water transport in the Dahl salt-sensitive rat CCD. *Am J Physiol*. 1991;260(4 pt 2):F471–F478.
35. Hawk CT, Schafer JA. Clonidine, but not bradykinin or ANP, inhibits Na+ and water transport in Dahl SS rat CCD. *Kidney Int*. 1993;44:30–35.
36. Kirchner KA, Crosby BA, Patel AR, Granger JP. Segmental chloride transport in the Dahl-S rat kidney during L-arginine administration. *J Am Soc Nephrol*. 1995;5:1567–1572.
37. Molony DA, Reeves WB, Andreoli TE. Na+:K+:2Cl- cotransport and the thick ascending limb. *Kidney Int*. 1989;36:418–426.
38. Hebert SC, Andreoli TE. Control of NaCl transport in the thick ascending limb. *Am J Physiol*. 1984;246(6 pt 2):F745–F756.
39. Hoagland KM, Flasch AK, Dahly-Vernon AJ, dos Santos EA, Knepper MA, Roman RJ. Elevated BSC-1 and ROMK expression in Dahl salt-sensitive rat kidneys. *Hypertension*. 2004;43:860–865.
40. Gunaratne R, Braucht DW, Rinschen MM, Chou CL, Hoffert JD, Pisitkun T, Knepper MA. Quantitative phosphoproteomic analysis reveals cAMP/vasopressin-dependent signaling pathways in native renal thick ascending limb cells. *Proc Natl Acad Sci USA*. 2010;107:15653–15658.
41. Rafiqi FH, Zuber AM, Glover M, Richardson C, Fleming S, Jovanović S, Jovanović A, O'Shaughnessy KM, Alessi DR. Role of the WNK-activated SPAK kinase in regulating blood pressure. *EMBO Mol Med*. 2010;2:63–75.
42. Yang CL, Angell J, Mitchell R, Ellison DH. WNK kinases regulate thiazide-sensitive Na-Cl cotransport. *J Clin Invest*. 2003;111:1039–1045.
43. Patel A, Layne S, Watts D, Kirchner KA. L-arginine administration normalizes pressure natriuresis in hypertensive Dahl rats. *Hypertension*. 1993;22:863–869.
44. Patel AR, Granger JP, Kirchner KA. L-arginine improves transmission of perfusion pressure to the renal interstitium in Dahl salt-sensitive rats. *Am J Physiol*. 1994;266(6 pt 2):R1730–R1735.

Novelty and Significance

What Is New?

- This study is the first to compare surface:total NKCC2 ratio and NKCC2 phosphorylation in DSS rats and Dahl salt-resistant rats on a normal salt diet.

What Is Relevant?

- Impaired trafficking and phosphorylation of NKCC2 may result in higher activity in DSS rats, thereby contributing to NaCl retention and hypertension in DSS rats and human salt-sensitive patients.

Summary

- Surface:total ratio of NKCC2 is higher in DSS rats.
- NKCC2 phosphorylation is enhanced in DSS rats.
- STE20- and SPS1-related proline and alanine-rich kinases/oxidative stress-responsive kinase 1 phosphorylation is increased in DSS rats.

Hyperphosphorylation of Na-K-2Cl Cotransporter in Thick Ascending Limbs of Dahl Salt-Sensitive Rats

Gustavo R. Ares, Mohammed Z. Haque, Eric Delpire and Pablo A. Ortiz

Hypertension. 2012;60:1464-1470; originally published online October 29, 2012;
doi: 10.1161/HYPERTENSIONAHA.112.202101
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0194-911X. Online ISSN: 1524-4563