Aldosterone acutely stimulates NCC activity via a SPAK-mediated pathway

Benjamin Ko,1 Abinash C. Mistry,2 Lauren Hanson,1 Rickta Mallick,2 Brandi M. Wynne,2 Tiffany L. Thai,2 James L. Bailey,2 Janet D. Klein,2,4 and Robert S. Hoover2,3,4

1Department of Medicine, University of Chicago, Chicago, Illinois; 2Division of Nephrology, Department of Medicine, Emory University, Atlanta, Georgia; 3Research Service, Atlanta Veterans Administration Medical Center, Decatur, Georgia; and 4Department of Physiology, Emory University, Atlanta, Georgia

Submitted 25 January 2013; accepted in final form 30 May 2013

Ko B, Mistry AC, Hanson L, Mallick R, Wynne BM, Thai TL, Bailey JL, Klein JD, Hoover RS. Aldosterone acutely stimulates NCC activity via a SPAK-mediated pathway. Am J Physiol Renal Physiol 305: F645–F652, 2013. First published June 5, 2013; doi:10.1152/ajprenal.00053.2013.—Hypertension is a leading cause of morbidity and mortality worldwide, and disordered sodium balance has long been implicated in its pathogenesis. Aldosterone is perhaps the key regulator of sodium balance and thus blood pressure. The sodium chloride cotransporter (NCC) in the distal convoluted tubule of the kidney is a major site of sodium reabsorption and plays a key role in blood pressure regulation. Chronic exposure to aldosterone increases NCC protein expression and function. However, more acute effects of aldosterone on NCC are unknown. In our salt-abundant modern society where chronic salt deprivation is rare, understanding the acute effects of aldosterone is critical. Here, we examined the acute effects (12–36 h) of aldosterone on NCC in the rodent kidney and in a mouse distal convoluted tubule cell line. Studies demonstrated that aldosterone acutely stimulated NCC activity and phosphorylation without affecting total NCC abundance or surface expression. This effect was dependent upon the presence of the mineralocorticoid receptor and serum- and glucocorticoid-regulated kinase 1 (SGK1). Furthermore, STE20/SPS-1-related proline/alanine-rich kinase (SPAK) phosphorylation also increased, and gene silencing of SPAK eliminated the effect of aldosterone on NCC activity. Aldosterone administration via a minipump in adenalec-tomized rodents confirmed an increase in NCC phosphorylation without a change in NCC total protein. These data indicate that acute aldosterone-induced SPAK-dependent phosphorylation of NCC increases individual transporter activity.

NCC; aldosterone; SPAK; WNK4

HYPERTENSION IS A DISEASE that affects nearly one-third of all American adults and billions worldwide (3a). The renin-angiotensin-aldosterone system (RAAS) is a major determinant of total body volume and thus blood pressure. It is now recognized that derangements of this system play a major role in the pathophysiology of hypertension. Aldosterone primarily exerts its influence on blood pressure homeostasis by regulating salt reabsorption in the distal tubule of the mammalian kidney. While the epithelial sodium channel (ENaC) has long been recognized as an effector of the RAAS, we know now that the sodium chloride cotransporter (NCC) in the distal convoluted tubule (DCT) is also a key effector of the RAAS, being stimulated by both angiotensin II and aldosterone (2, 5, 18, 24, 28, 36, 38, 44–46).

NCC is a key determinant of sodium balance, reabsorbing 5–10% of the total filtered load of sodium in the kidney (13). Disturbances in NCC function are characterized by disordered total body volume regulation and therefore disturbed blood pressure regulation. This is evidenced not only by the efficacy of thiazide diuretics but also by the hypotension seen with loss of NCC function (Gitelman’s syndrome) and the hypertension seen with increases in NCC activity (familial hyperkalemic hypertension or FHHt) (6, 14, 16, 32, 39, 49).

The role of aldosterone in the regulation of the cotransporter has evolved over the last decade. In studies of chronic salt restriction or chronic aldosterone infusions lasting anywhere from 3 to 8 days, NCC protein abundance and function increases significantly (18, 28, 46). While it has been reported that there are changes in NCC mRNA at 14 days (24), there is not a change in NCC message during this 3- to 8-day time frame (18, 28, 46). Therefore, these changes are largely mediated by posttranslational changes in NCC. The key posttranslational regulators of NCC are to be phosphorylation and ubiquitylation. In fact, regulation of NCC by chronic aldosterone exposure seems to involve both of these processes. NEDD4-2, a protein known to be regulated by aldosterone, appears to ubiquitylate NCC, resulting in decreased total NCC protein (2). Additionally, chronic aldosterone or a low-salt diet results in increased phosphorylation of NCC (5, 44). This process is mediated by the kinases STE20/SPS-1-related proline/alanine rich kinase (SPAK) and oxidative stress-responsive 1 (OSR1) which directly phosphorylate NCC in the amino terminus at threonine (Thr) 55, and Thr60 in human NCC (corresponding to Thr 53 and 58, respectively, in rats) (34, 42, 47, 48), resulting in increased NCC activity (1, 12, 30, 47). The WNK [With-No-Lysine (K)] kinases WNK1 and WNK4 appear to act as upstream modulators of these kinases. These key studies have formed the basis of our knowledge about aldosterone’s effects on NCC.

While studies examining the long-term effect of aldosterone provide critical knowledge of the physiology of salt handling in the kidney, they are modeling the normal physiological state of the low-salt diet hunter-gatherer societies of ancient humans. Today we ingest three to five times the salt that our ancestors did (21, 22). In this environment, a week of even moderate salt deprivation is a more common occurrence. Therefore, understanding the physiology of the effects of aldosterone in this time frame is very important. However, the acute effects of aldosterone on NCC remain largely unexplored. We know that there is no significant change in NCC total protein at 24 h of salt restriction (28), but the activity, surface expression, and phosphorylation of the cotransporter have not been examined.

The work discussed herein closely examines the acute (12–36 h) effects of aldosterone stimulation upon NCC activity and the mechanisms by which they occur. To accomplish this,
a mammalian DCT cell model with robust NCC activity and native expression of DCT proteins was used (20). Using this unique model, we demonstrated that aldosterone acutely stimulated NCC activity without affecting total NCC abundance or NCC surface expression. NCC phosphorylation, however, did increase. In vivo studies confirmed the increase in phosphorylated NCC without an effect on total NCC. Chemical inhibition and RNA interference studies demonstrated that the effect of aldosterone on NCC activity was dependent upon serum- and glucocorticoid-regulated kinase 1 (SGK1) and SPAK, respectively. These studies indicate that the increased NCC activity induced by aldosterone is due to an increase in individual transporter activity mediated by SPAK phosphorylation in the absence of increased abundance or surface expression.

MATERIALS AND METHODS

Materials. Materials were purchased from Sigma-Aldrich (St. Louis, MO), unless stated otherwise.

Cell culture and treatments. mDCT15 cells were plated on cell culture dishes and grown in growth medium containing a 50:50 mix of DMEM/F12, 5% heat-inactivated FBS, and 1% penicillin/streptomycin/neomycin (P/S/N) at 37°C. Experiments were conducted when the cells reached 90–95% confluence.

Assessment of NCC function in mDCT15 cells. mDCT15 cells were seeded in 12-well plates and prepared as described. The cells were then incubated in a serum-free growth media (Opti-Mem) for 24 h before being assayed. Cells were then treated with aldosterone or vehicle (DMSO) for the indicated times and concentrations. Thirty minutes before uptake, 0.1 mM metolazone was added to the media. GS650394 (100 nM) was added to the media for 24 h before uptake in studies using GS6 50394. The medium was then changed to a 22Na-containing medium (140 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 5 mM HEPES/Tris pH 7.4, 1 mM amiloride, 0.1 mM bumetanide, 0.1 mM benzamil, 1 mM ouabain, and 1 μCi/ml of 22Na) with or without thiadizide (0.1 mM metolazone) and incubated for 20 min. Tracer uptake was then stopped via washes with ice-cold wash buffer. Cells were subsequently lysed with 0.1% SDS. Radioactivity was measured via liquid scintillation, and protein concentrations of the lysates were determined by a BCA protein assay kit. The appropriate amount of each sample was analyzed for statistical significance using a paired Student’s t-test or Mann-Whitney Rank Sum test where appropriate. A P value of <0.05 was taken as statistically significant.

RESULTS

Short-term treatment with aldosterone stimulates NCC activity. To assess the early effects of aldosterone on NCC activity, mDCT15 cells were preincubated in serum-free media

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for 24 h and then treated with aldosterone before NCC activity was determined by radionucleotide uptake. Short-term treatment with 100 nM aldosterone significantly increased NCC activity (Fig. 1A). NCC activity was increased by 30% at 12 h, 52% at 24 h, and 56% at 36 h (Fig. 1A, n = 6, P < 0.05). Following NCC activity with increasing concentrations of aldosterone (24-h incubation) demonstrates a significant increase in NCC activity at aldosterone concentrations as low as 10 nM without significant increases in NCC activity after the aldosterone concentration exceeds 100 nM (Fig. 1B, n = 4, P < 0.05). This clear dose-response relationship reinforces that aldosterone exerts an early effect on NCC activity.

**Aldosterone acts on NCC via the mineralocorticoid receptor and SGK1.** The dose-response relationship of NCC activity and aldosterone shows no significant increase in NCC activity with aldosterone concentrations >100 nM (Fig. 1B). Mineralocorticoid effects can be mediated by either the glucocorticoid receptor (GR) or the mineralocorticoid receptor (MR). However, effects due to MR generally plateau at 100 nM aldosterone while glucocorticoid-mediated ones may continue to display enhanced responses at higher concentrations (11). This suggests that the mDCT15 NCC response to aldosterone stimulation is mediated by MR. To test this hypothesis, the presence of MR in mDCT15 cells was confirmed via Western blotting (Fig. 2A). Thiazide-sensitive $^{22}$Na$^{+}$ uptake was then measured in response to aldosterone as before but now in the presence of the MR antagonist spironolactone or the GR antagonist RU-486. As shown in Fig. 2B, neither 10 nM spironolactone nor 20 μM RU-486 affected baseline NCC activity. Spironolactone-treated groups, however, did not display increased NCC activity in response to aldosterone stimulation. RU-486 did not impede aldosterone-stimulated NCC activity. This indicates that aldosterone’s effects on NCC activity are primarily mediated via MR in mDCT15 cells,

![Fig. 1. Effect of aldosterone on sodium chloride cotransporter (NCC) activity.](image)

**Fig. 1. Effect of aldosterone on sodium chloride cotransporter (NCC) activity.** A: mDCT15 cells were treated with 100 nM aldosterone for the indicated times before incubating in uptake medium with $^{22}$Na$^{+}$ and either vehicle or 1 mM metolazone. Radioactive uptake was determined as in MATERIALS AND METHODS. Thiazide-sensitive uptake was calculated as the difference in radiotracer uptake between the metolazone-containing and the metolazone-free groups. *P < 0.05; n = 6. B: mDCT15 cells were incubated in aldosterone at the indicated concentrations for 24 h before determination of radiotracer uptake as outlined above. *P < 0.05; n = 4.

![Fig. 2. Effect of mineralocorticoid receptor (MR) antagonism on aldosterone-induced NCC activity.](image)

**Fig. 2. Effect of mineralocorticoid receptor (MR) antagonism on aldosterone-induced NCC activity.** A: mDCT15 cells were lysed and immunoblotted for MR. Kidney cortex is shown as a positive control. A representative blot is shown. B: mDCT15 cells were treated with 100 nM aldosterone (A) for 24 h in the presence or absence of the MR antagonist spironolactone (S) or the GR antagonist RU-486 (R). *P < 0.05; n = 4. C: mDCT15 cells were treated with 100 nM aldosterone (A) or vehicle [V(A)] for 24 h in the presence or absence of the SGK1 antagonist GSK 650394 (I) or vehicle [V(I)]. *P < 0.05; n = 4. #P < 0.05.
while GR exerts a minimal effect. MR activation is classically associated with SGK1 activation, and so aldosterone dependence on SGK1 was assessed by measuring thiazide-sensitive \(^{22}\text{Na}\) uptakes in response to aldosterone in the presence or absence of the SGK1 inhibitor GSK 650394. GSK 650394 (100 nM) reduced baseline NCC activity by 21%.

mDCT15 cells treated with the SGK1 inhibitor displayed no significant increase in NCC activity with aldosterone stimulation compared with control. These data confirm that acute aldosterone effects on NCC are mediated primarily by the MR and SGK1.

**Short-term aldosterone treatment does not increase NCC abundance or distribution.** Previous reports examining longer term aldosterone effects have demonstrated an increase in total cellular NCC abundance in response to low-salt and/or high-aldosterone states (18, 28). Immunoblotting was performed to assess for any changes in total NCC abundance during short-term incubations, specifically after 24 h of aldosterone treatment. As shown in Fig. 3, no change in total NCC protein was observed at 24 h. Cell surface biotinylation was used to assess for any changes in surface expressed NCC protein, and no change in cell surface-expressed NCC was seen after 24 h of aldosterone exposure. Since aldosterone sharply increased NCC activity without a change in total protein abundance or surface expression, this suggests that short-term aldosterone exposure enhances NCC activity not by changing protein abundance or distribution but instead primarily via increasing individual NCC transporter activity.

**Aldosterone increases NCC phosphorylation.** To further examine mechanisms leading to this aldosterone-mediated change in NCC activity, NCC phosphorylation was examined. To confirm that any associated changes in NCC phosphorylation are associated with our observed acute increases in NCC activity, a time course of aldosterone stimulation was performed at 12, 24, and 36 h of aldosterone stimulation and surface expression, total protein, and phosphorylation were assessed. As demonstrated previously, aldosterone stimulation had no statistically significant effect upon NCC distribution or abundance at the 12- and 24-h time points, with a trend toward increased NCC surface expression at 36 h (Fig. 4A).

Fig. 3. Effect of aldosterone on NCC surface expression and total abundance. mDCT15 cells were grown to confluence, then treated with 100 nM aldosterone for 24 h before labeling with biotin at 4°C. A: biotinylated (cell surface) proteins were recovered using streptavidin-agarose and immunoblotted for NCC. Representative blot is shown with accompanying densitometry (C = control 0 h, 24C = control 24 h, 24A = aldol 24 h). B: total cell lysate as in A immunoblotted for NCC. Representative blot is shown with accompanying densitometry.

Fig. 4. Time course of aldosterone’s effects on NCC surface expression, total abundance, and NCC phosphorylation. mDCT15 cells were grown to confluence, then treated with 100 nM aldosterone for the indicated times. A: cells were labeled with biotin at 4°C. Biotinylated (cell surface) proteins were recovered using streptavidin-agarose and immunoblotted for NCC. Representative blot is shown with accompanying densitometry (C = control 0 h, 24C = control 24 h, 24A = aldol 24 h). B: cell lysate was then immunoblotted for NCC, phospho-NCC (NCC-T53), and actin. Representative immunoblot and densitometry are shown. *P < 0.05; n = 6.
Metric analysis of immunoblots revealed that NCC phosphorylation (phospho/total ratio) was increased approximately threefold at 12, 24, and 36 h. At each time point, the phosphorylation of NCC increased compared with vehicle control (Fig. 4B, n/H11005/6, P/H11021/0.05). While there is a trend toward increased phosphorylation over time, this did not reach statistical significance. Total NCC and actin were not significantly different over this time frame.

To confirm the aldosterone-induced increase in NCC phosphorylation without a change in total NCC protein expression seen at the cellular level, we decided to examine the effect of short-term aldosterone on NCC at the whole-animal level. Here, we used adrenalectomized rats and administered aldosterone or vehicle (25% DMSO) via osmotic minipumps for 24 h. Kidney cortex was then harvested and analyzed via immunoblotting. Densitometry analysis showed that the rats receiving aldosterone demonstrated no change in total NCC or actin compared with control (Fig. 5A). However, there was an almost threefold change in phospho-NCC (T53) (densitometry in Fig. 5B, n/H11005/10, P/H11021/0.01). These data confirm that aldosterone induces NCC phosphorylation in the whole organism as well.

**SPAK mediates aldosterone’s actions on NCC.** Aldosterone’s enhancement of NCC phosphorylation at threonine 53 suggests that it stimulates a kinase which phosphorylates NCC at threonine 53. Because of their known involvement in NCC regulation, the kinases SPAK/OSR1 are likely candidates to key this signaling cascade. SPAK/OSR1 phosphorylation was therefore assessed via immunoblotting using phospho-specific antibodies for SPAK or OSR1. SPAK 373 phosphorylation was increased in aldosterone-stimulated mDCT15 cells compared with control (Fig. 6A, 35 ± 14% at 24 h as measured by densitometry, P < 0.05). Similarly, OSR1 displayed an increase in phosphorylation, although the total abundance of OSR1 is lower than that of SPAK (Fig. 6B).

Given that SPAK expression predominates over OSR1 in our model, SPAK protein expression was decreased via RNA interference to assess whether signaling via SPAK plays a crucial role in NCC phosphorylation and activation. RNA interference resulted in a 68 ± 7% decrease in SPAK protein expression compared with nontargeting controls as measured by Western blotting and densitometry (Fig. 7A, n = 3, P < 0.05). Specificity of RNA interference for SPAK was confirmed via immunoblotting for OSR1, and OSR1 protein expression was unaffected in these cells (Fig. 7A). Gene knockdown of SPAK decreased baseline NCC activity in these cells by 36 ± 3% compared with control (Fig. 7B, n = 4, P < 0.01). Stimulation of these SPAK knockdown cells with aldosterone had virtually no effect on NCC activity (6 ± 3% increase after 24-h aldosterone treatment in short hairpin RNA SPAK groups vs. 52 ± 5% increase after aldosterone treatment in control groups, n = 4, P < 0.01). This provides clear evidence that
aldosterone acutely increases NCC activity without changing NCC abundance or distribution via a pathway involving SPAK phosphorylation.

DISCUSSION

Although aldosterone has traditionally been thought to mediate its effects on sodium reabsorption primarily through regulation of the epithelial sodium channel (ENaC), the first definitive studies linking aldosterone to activation of NCC were published more than a decade ago (46). These and subsequent studies indicated that chronic aldosterone exposure (greater than 72 h) increases NCC activity in the DCT through increases in NCC protein expression. Studies in rats, dogs, and humans have demonstrated that aldosterone acutely causes a sharp decrease in sodium excretion in the 2- to 24-h time period after administration (17, 26–29, 43). However, acute effects of aldosterone on NCC have not been determined.

Here, the role of aldosterone in acute NCC effects was investigated in both the mDCT15 cell line and adrenalectomized rats given an aldosterone infusion. Significant increases were seen in NCC activity in response to aldosterone stimulation as early as 12 h and continued to increase through 36 h. This clearly defines a role for aldosterone in early RAAS effects on NCC. By first immunoblotting for MR and then performing NCC activity studies with aldosterone in the presence of steroid receptor antagonists, the role of MR in mediating this effect was confirmed. The dependence of SGK1 on these effects was then demonstrated using an SGK1 inhibitor. Aldosterone treatment had no significant effect on NCC activity in mDCT15 cells treated with an SGK1 inhibitor. Cell surface and total cellular NCC immunoblots of mDCT15 cells revealed that aldosterone did not affect either NCC abundance or surface expression at 24 h (Fig. 3). Additionally, adrenalectomized rats administered aldosterone for 24 h showed no change in NCC total protein (Fig. 4A). This is consistent with both whole-animal data (28) and cell data in the precursor mDCT cell line (15) indicating no change in NCC abundance with 24-h low-salt or aldosterone stimulation. However, primarily because of the lack of a mammalian cell model than can measure activity of NCC and the difficulty of DCT microperefusion, acute effects of aldosterone on NCC activity have not been reported. Utilizing this new model, we have demonstrated that the stimulation of NCC activity by aldosterone appears to be mediated by SPAK/OSR1-dependent phosphorylation of NCC, resulting in increased activity of individual NCC proteins. Indeed, this mechanism of SPAK/OSR1 stimulation of NCC without changes in total protein or surface expression has been reported by others (9, 33). In addition to the SPAK dependence of the aldosterone effect, decreasing SPAK abundance decreased baseline function of the cotransporter (Fig. 7B). This reemphasizes the critical role of SPAK phosphorylation in the regulation of NCC.

The likely potential upstream partners of SPAK in this signaling cascade are the WNK kinases, commonly associated with OSR1/SPA K. However, while all investigators acknowledge that WNKs are important regulators of NCC, mechanisms of that regulation remain controversial. WNK4 in particular has been demonstrated to both stimulate and inhibit NCC depending on the model system and physiological stimuli (3, 25, 37, 40, 50–52). Chronic low-salt diets appear to have no effect on WNK1 protein abundance (5, 10). However, some studies have shown changes in WNK4 abundance and message while others have not (5, 24, 31). Studies of the effects of aldosterone on WNK4 have also yielded conflicting results (41, 44). To our knowledge, studies of the effects of aldosterone administration on WNK1 abundance have not been reported. It is important to note, however, that even if protein abundance of WNKs was unchanged it would not preclude WNK1 or WNK4 involvement. Indeed, SGK1, a known mediator for aldosterone, has been shown to phosphorylate WNK4 and also is known to both regulate and be regulated by WNKs (4, 36). WNK4 and/or WNK1 could therefore mediate their actions via a phosphorylation event rather than by an increase in protein abundance. Given that we do not observe a change in NCC surface expression, NEDD4-2 seems a less likely partner for SGK1’s actions upon NCC, but conceivably, WNK4, WNK1, both, or neither could mediate our observed effect as the intermediary between SGK1 and SPAK. In the case of WNK4, we would hypothesize that SGK1 phosphorylates WNK4, leading to release of inhibition of NCC activity (via an increase in SPAK phosphorylation), as has been previously suggested in the literature (35, 36). Conversely, phosphorylation of WNK1 by SGK1 could then lead to enhanced SPAK phosphorylation. Adding to the complexity, WNK3 has recently been reported to antagonize the action of
Nedd4-2 on NCC, making WNK3 another possible partner for aldosterone (23). Because of the enormous complexity of these issues, this represents a fertile area for future studies.

We would now hypothesize that aldosterone increases the activity of NCC within hours of its release from the adrenal gland via activation of SPAK, resulting in increased “in situ” cotransporter activity. This acute effect does not involve changes in protein expression and is thus potentially quickly reversed with suppression of aldosterone release by a salt load. While understanding the physiology of the chronic effects of aldosterone is important, understanding the physiology of aldosterone effects in this 12- to 36-h period could be especially important in the salt-abundant modern world we live in. This environment is much different from the low-salt hunter-gatherer environment with presumably chronically high aldosterone levels in which our genomes evolved. In fact, it is estimated that the diet of the typical hunter gatherer consisted of <1 g of sodium a day compared with the 3.4 g of sodium we now consume a day (21, 22). This low-salt diet has been the normal physiological state for 99% of the 2.5 million-year existence of the Homo genus. Only 10,000 years ago did we begin to transition to the salt-abundant world that we now live in (21, 22). In modern Western society, where hypertension is predominantly found, 99% of adults have >1.5 g of sodium intake a day, and 92% consumed >2.3 g (7). In this environment, a week of even moderate salt deprivation is rare, and 12–36 h of salt restriction is a more common occurrence. Therefore, a system which can modulate salt reabsorption rapidly with rapid changes in salt intake would be critical.

Then, if the salt deficit persists, the chronic mechanisms of salt preservation begin. We would hypothesize that somewhere between 36 and 72 h the mechanism of NCC stimulation by aldosterone switches to an increase in the abundance of cotransporter. Work by Arroyo, et al. (2) indicates that the chronic stimulation of NCC protein abundance by aldosterone may potentially involve decreased ubiquitination of NCC by Nedd4-2. This mechanism would be consistent with the change in protein expression of NCC without a change in message seen with 72 h of salt restriction as reported by Masilimani et al. (28). It has now been reported that extended periods (2 wk) of a low-salt diet may result in an increase in NCC message as well as protein (24). Thus the stimulatory effect of aldosterone on NCC may actually be triphasic: Acutely, aldosterone increases function without changing abundance; then, it changes abundance without changing message, and finally after extended exposure perhaps it changes message and abundance. The quick onset and potential quick reversibility of the acute aldosterone effect on NCC would allow for a fast response to volume depletion and salt deprivation while preventing prolonged inappropriate hyperactivity of the cotransporter (as might be seen with changes in protein expression) in response to a short period of salt deprivation. The more robust chronic aldosterone effect would be critical to retaining salt in chronic salt deprivation as was quite common in the hunter-gatherer societies of our ancestors.

In sum, this work establishes the acute effects of aldosterone on NCC activity and phosphorylation in both a cellular and whole animal model. Aldosterone was found to sharply increase NCC activity without affecting total NCC abundance or NCC surface expression. NCC phosphorylation and SPAK phosphorylation did increase with the effect on NCC activity, with the effect dependent upon SGK1 as well as SPAK. Therefore, we report that aldosterone acutely stimulates increased NCC activity due to an increase in individual transporter activity mediated by SPAK.

ACKNOWLEDGMENTS

We thank Eugene Chang, Mark Musch, and David Pearce for advice and discussions.

GRANTS

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants R01 DK-085097 (to R. S. Hoover), K08 DK081728 (to B. Ko), T32 DK07656 (to T. L. Thai), and R01 DK41707 (to J. D. Klein) as well as via the Research Service, Atlanta VA Medical Center, Decatur, GA.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: B.K., J.D.K., and R.S.H. provided conception and design of research; B.K., A.C.M., L.N.H., R.M., B.M.W., T.L.T., and J.L.B. performed experiments; B.K., A.C.M., L.N.H., R.M., and R.S.H. analyzed data; B.K. and R.S.H. interpreted results of experiments; B.K. drafted manuscript; B.K. and R.S.H. edited and revised manuscript; B.K. and R.S.H. approved final version of manuscript.

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