Calcineurin inhibitors block sodium-chloride cotransporter dephosphorylation in response to high potassium intake

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Dietary potassium intake is inversely related to blood pressure and mortality. Moreover, the sodium-chloride cotransporter (NCC) plays an important role in blood pressure regulation and urinary potassium excretion in response to potassium intake. Previously, it was shown that NCC is activated by the WNK4-SPAK cascade and dephosphorylated by protein phosphatase. However, the mechanism of NCC regulation with acute potassium intake is still unclear. To identify the molecular mechanism of NCC regulation in response to potassium intake, we used adult C57BL/6 mice fed a 1.7% potassium solution by oral gavage. We confirmed that acute potassium load rapidly dephosphorylated NCC, which was not dependent on the accompanying anions. Mice were treated with tacrolimus (calcineurin inhibitor) and W7 (calmodulin inhibitor) before the oral potassium loads. Dephosphorylation of NCC induced by potassium was significantly inhibited by both tacrolimus and W7 treatment. There was no significant difference in WNK4, OSR1, and SPAK expression after high potassium intake, even after tacrolimus and W7 treatment. Another phosphatase, protein phosphatase 1, and its endogenous inhibitor I-1 did not show a significant change after potassium intake. Hyperkaliuria, induced by high potassium intake, was significantly suppressed by tacrolimus treatment. Thus, calcineurin is activated by an acute potassium load, which rapidly dephosphorylates NCC, leading to increased urinary potassium excretion.

Kidney International (2016) ■, ■–■; http://dx.doi.org/10.1016/j.kint.2016.09.001

KEYWORDS: calcineurin; phosphatase; potassium; sodium-chloride cotransporter

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Received 13 May 2016; revised 25 August 2016; accepted 1 September 2016

Potassium (K^+) intake is important for human health, and it is well-known that higher K^+ intake is associated with lower mortality and blood pressure (BP).[1–3] On the other hand, acute hyperkalemia can be fatal. Therefore, it is important to elucidate the mechanism of BP reduction and K^+ excretion after K^+ intake. Sodium-chloride cotransporter (NCC) is expressed in distal convoluted tubules (DCTs) and plays an important role in the regulation of BP and K^+ homeostasis. Altering sodium (Na^+) delivery to the connecting tubules and cortical collecting ducts, which are key sites of K^+ secretion, regulates K^+ excretion.[4]

The molecular mechanism of NCC regulation has been investigated through the study of pseudohypoaldosteronism type II—Gordon syndrome, which is characterized by hereditary hypertension with hyperkalemia and metabolic acidosis. NCC is phosphorylated and activated by Ste20-related proline- and alanine-rich kinase (SPAK) kinase, and SPAK is regulated by Kelch-like protein 3 (KLHL3)- with no lysine kinase 4 (WNK4) cascade.[5,6]

In previous animal studies, a low-K^+ diet caused increases in the amount and phosphorylation of NCC[4,7–13] as well as elevation of BP.[4,13] Elevation of BP with a low-K^+ diet was dependent on NCC because $NCC^{-/-}$ mice did not show an elevation of BP with a low-K^+ diet.[4] A low-K^+ diet also increased total and phosphorylated SPAK[4,10,12,13] and WNK4.[4,13] The $WNK4^{-/-}$ and $SPAK^{-/-}$ mice showed either no increase or only a blunted increase in phosphorylated NCC (pNCC) in response to a low-K^+ diet, respectively.[4,10,12]

These results indicate that phosphorylation of NCC with a low-K^+ diet is dependent on WNK4-SPAK kinases. In recent years, it has been found that WNK1 has a chloride (Cl^-)-binding motif that affects WNK1 autophosphorylation.[14] Other WNKs also have a Cl^--sensing domain that is homologous to the WNK1 domain.[9] A change in plasma K^+ level affects the membrane potential of DCT cells, thereby altering their intracellular Cl^- concentration. Therefore, WNKs are thought to be K^+ sensors.[4,9] Our previous study of COS7 cells highlighted the importance of K^+ in stimulating the WNK cascade in low-K^+ conditions.[15]

As for K^+ loading, there are various experimental protocols and results on the effect of high K^+ on NCC. Dietary intake, rapid oral gavage, and i.v. administration of KCl decreased total and pNCC.[7,9,11,16–18] Rapid oral gavage of $KHCO_3$ also induced dephosphorylation of NCC.[16] However, a high-K^+

diet of K^+-citrate increased pNCC.[8,13] In addition to NCC, the effect of high K^+ on WNK-SPAK kinase is also controversial. Rengarajan *et al.*[17] showed a slight decrease of phosphorylated SPAK in a high-KCl diet; however, other groups showed an increase in WNK4 and phosphorylated SPAK[18] as well as an increase in WNK4 mRNA level[19] in a high-K^+ diet.

Other than WNK4-SPAK kinases, phosphatases are thought to regulate the phosphorylation of NCC as a counterbalance to the kinases. Indeed, in previous studies, NCC was reported to be dephosphorylated by calcinurin,[20] protein phosphatase 1 (PP1),[21] and PP4.[22] Calcineurin is known as a calcium- and calmodulin-dependent protein serine/threonine phosphate, termed protein phosphatase 2B.[23]

Calcineurin inhibitors, such as tacrolimus and cyclosporine, are immunosuppressive drugs that have side effects such as hyperkalemia and hypertension. Tacrolimus and cyclosporine were reported to increase the abundance of NCC.[24,25] When the tacrolimus-binding protein (FKBP12) was specifically knocked out in the mouse kidney, the tacrolimus-induced NCC phosphorylation was not observed.[20] Another phosphatase, PP1, was also thought to regulate NCC. Protein phosphatase inhibitor-1 (I-1), known as an endogenous inhibitor of PP1, was identified as a DCT-enriched gene product by microarray analysis of mouse DCT cells, and the I-1 knockout mouse experiment by Picard *et al.*[21] showed a decrease in pNCC and significantly lower arterial BP. These data suggested that phosphatases are involved in the normal regulation of NCC.

Seeking to identify the mechanism involved in the regulation of NCC with K^+ intake *in vivo*, we focused on the rapid decrease of NCC phosphorylation during acute K^+ load in mice. Our data showed that calcineurin was activated during acute K^+ load and dephosphorylated NCC.

RESULTS

Potassium-induced NCC dephosphorylation is not dependent on the anions in the acute phase

We first verified the change in NCC phosphorylation with acute oral K^+ administration. KCl, K^+-gluconate, or K^+-citrate was fed to mice with oral gavage, and we found that all potassium compounds significantly decreased NCC phosphorylation 15 minutes after oral gavage (Figure 1A).

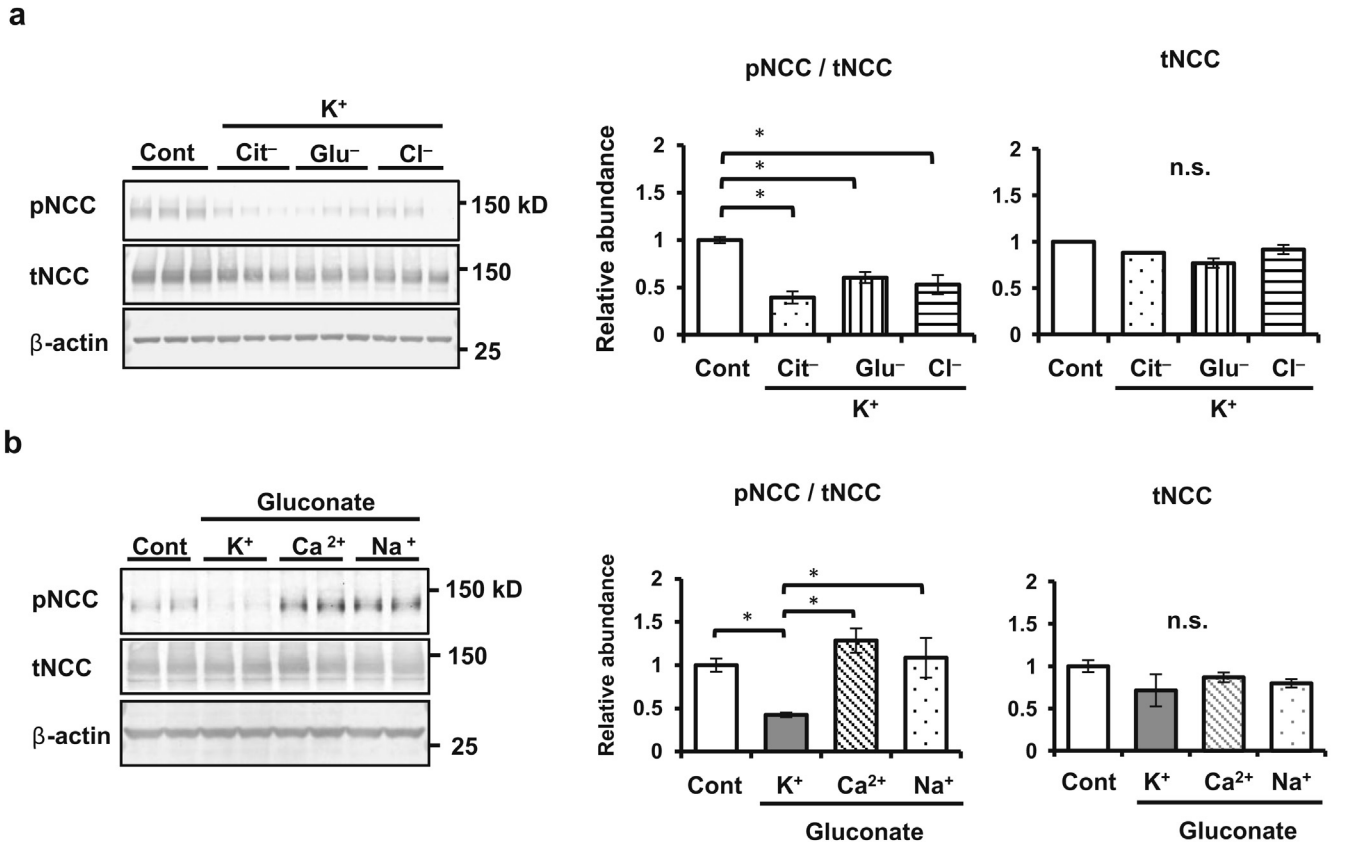

Figure 1 | Rapid K^+ infusion decreased NCC phosphorylation. (**a**) Immunoblots of mouse kidneys collected 15 minutes after oral gavage of K^+ solutions. All high K^+ solutions decreased NCC phosphorylation. Quantitative analysis of total and phosphorylated NCC in column graphs (N = 3). (**b**) Representative immunoblots of mouse kidneys 15 minutes after oral gavage of gluconate. An equivalent volume of calcium gluconate or sodium gluconate did not decrease phosphorylated NCC. Quantitative analysis of total and phosphorylated NCC in column graphs (N = 4). Ca^{2+}, calcium; Cit^-, citrate; Cl^-, chloride; Cont, control; Glu^-, gluconate; K^+, potassium; Na^+, sodium; NCC, sodium-chloride cotransporter; n.s., not significant; pNCC, phosphorylated sodium-chloride cotransporter; tNCC, total sodium-chloride cotransporter; SPAK, Ste20-related proline- and alanine-rich kinase; WNK4, With no lysine kinase 4. *$P < 0.05$.