Potassium Supplementation Prevents Sodium Chloride Cotransporter Stimulation During Angiotensin II Hypertension

Luciana C. Veiras, Jiyang Han, Donna L. Ralph, Alicia A. McDonough

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Abstract—Angiotensin II (AngII) hypertension increases distal tubule Na-Cl cotransporter (NCC) abundance and phosphorylation (NCCp), as well as epithelial Na⁺ channel abundance and activating cleavage. Acutely raising plasma [K⁺] by infusion or ingestion provokes a rapid decrease in NCCp that drives a compensatory kaliuresis. The first aim tested whether acutely raising plasma [K⁺] with a single 3-hour 2% potassium meal would lower NCCp in Sprague–Dawley rats after 14 days of AngII (400 ng/kg per minute). The potassium-rich meal neither decreased NCCp nor increased K⁺ excretion. AngII-infused rats exhibited lower plasma [K⁺] versus controls (3.6±0.2 versus 4.5±0.1 mmol/L; *P*<0.05), suggesting that AngII-mediated epithelial Na⁺ channel activation provokes K⁺ depletion. The second aim tested whether doubling dietary potassium intake from 1% (A1K) to 2% (A2K) would prevent K⁺ depletion during AngII infusion and, thus, prevent NCC accumulation. A2K-fed rats exhibited normal plasma [K⁺] and 2-fold higher K⁺ excretion and plasma [aldosterone] versus A1K. In A1K rats, NCC, NCCpS71, and NCCpT53 abundance increased 1.5- to 3-fold versus controls (*P*<0.05). The rise in NCC and NCCp abundance was prevented in the A2K rats, yet blood pressure did not significantly decrease. Epithelial Na⁺ channel subunit abundance and cleavage increased 1.5- to 3-fold in both A1K and A2K; ROMK (renal outer medulla K⁺ channel abundance) abundance was unaffected by AngII or dietary K⁺. In summary, the accumulation and phosphorylation of NCC seen during chronic AngII infusion hypertension is likely secondary to potassium deficiency driven by epithelial Na⁺ channel stimulation.

Key Words: aldosterone-sensitive distal nephron ■ angiotensin II hypertension ■ dietary potassium ■ hypokalemia ■ kaliuresis

The angiotensin II (AngII) infusion model of experimental hypertension has been implemented in thousands of studies and has provided insights into mechanisms that cause the resultant hypertension and cardiovascular injury.1–3 We and others have defined key intrarenal mechanisms controlling the blood pressure (BP) by profiling the regulation of sodium transporters expressed along the nephron. In brief, we found that AngII increases abundance and stimulates transporters beyond the macula densa, including the apically expressed cortical Na⁺K⁺-2Cl⁻ cotransporter (NKCC2),4 Na⁺-Cl cotransporter (NCC), and both cortical and medullary epithelial Na⁺ channel (ENaC), whereas the resultant hypertension depresses the abundance and activation of cortical and medullary Na⁺H exchanger and medullary NKCC2.5 In rodent models that exhibit blunted hypertensive responses to AngII, transporter profiling reveals blunting of either distal transporter activation or proximal transporter inhibition.6,4 Even so, a direct association between distal cotransporter abundance and hypertension is not evident in mice overexpressing NCC, indicating the importance of integrating multiple mechanisms along the entire nephron.9 Chronic AngII regulation of NCC and its phosphorylation have been attributed to the stimulation of a WNK-SPAK (with-no-lysine kinase-Ste20/SPS-1–related proline–alanine rich kinase) kinase cascade.10,11 Indeed, our own studies show that renal cortical SPAK is stimulated during chronic AngII infusion hypertension, and that if intrarenal production of AngII is prevented, both the NCC and SPAK stimulation are blocked, and the rise in BP is blunted.4,8

The beneficial effect of raising dietary potassium to lower BP is indicated in both epidemiology and interventional studies in humans and laboratory animals.12–14 It is now evident that the BP-lowering property may, at least in part, be linked to renal responses activated to excrete potassium: raising plasma potassium acutely (via a potassium-rich meal, oral gavage, or potassium infusion) drives a rapid decrease in NCC phosphorylation and activity, which lowers Na⁺ reabsorption by the...
distal convoluted tubule (DCT) NCC and drives Na⁺ and volume downstream for reabsorption by cortical collecting duct ENaC, which provokes K⁺ secretion by renal outer medulla K⁺ channel (ROMK) and flow sensitive large conductance Ca²⁺-activated K⁺ (BK) channels in the same region, the impact of which is to match K⁺ excretion to K⁺ intake.15,16 Thus, an increase in potassium input acts like a thiazide diuretic to suppress NCC activity that not only raises K⁺ excretion but also decreases Na⁺ reabsorption by NCC, which can lower BP set point.14,17,18 In fact, evidence suggests that homeostatic control of potassium regulation has a higher hierarchical importance than control of sodium and volume.19-21

During AngII-dependent hypertension, DCT NCC phosphorylation (NCCp) increases 2- to 4-fold as does NCC-mediated Na⁺ reabsorption.4,8 Motivated by these findings, we tested the hypothesis that the NCCp, elevated by AngII infusion hypertension, could be reversed by raising potassium intake. We discovered that AngII infusion per se provokes a kaliuresis and potassium depletion, most likely secondary to ENaC activation: Mamenko et al have determined that AngII infusion hypertension increases ENaC membrane abundance and activation far above the physiological stimulation observed when animals were fed near-zero salt diet.22 In support of the connection between inappropriate stimulation of ENaC by AngII driving potassium loss and NCC activation, we discovered that doubling potassium intake normalizes potassium homeostasis and prevents the rise in NCC and NCCp observed in response to AngII infusion.

Concise Methods
Detailed Methods are available in the online-only Data Supplement.

Animal Protocols
All animal procedures were approved by the Institutional Animal Care and Use Committee of the Keck School of Medicine of the University of Southern California. Male Sprague–Dawley rats (225–250 g body weight; Harlan Laboratories, San Diego, CA) were anesthetized intramuscularly with 200 μL of 1:1 volume ratio of ketamine (Phoenix Pharmaceuticals, St. Joseph, MO) and xylazine (Lloyd laboratories, Shenandoah, IA) and randomized to 2 groups, implanted with osmotic minipumps (Alzet, model 2002, Cupertino, CA) subcutaneously to deliver AngII (400 ng/kg per minute,14 days) were fasted overnight and then fed a 0%K meal (A+0K) or 2%K meal (A+2K), and non–AngII infused rats were fed the 0%K meal (C+0K). A+0K, or 2%K meal (C+2K) or C+0K groups consumed 14±2, 10±1, and 14±1 g/3 hours, respectively. Unexpectedly, plasma [K⁺] was significantly lower in A+0K versus C+0K-fed rats (3.6±0.2 versus 4.5±0.1 mmol/L; Figure 1). The protocol was performed twice (and data pooled) with the same lower plasma K⁺ detected in A+0K versus C+0K. This decrease was not detected in our previous studies of AngII-infused rats,4 likely because those rats were fed ad libitum the night before plasma was collected, preserving K⁺ intake, whereas in this series, rats were fasted overnight during which they lost more K⁺ than they took in (K⁺output<K⁺intake). Thus, the overnight potassium deprivation unmasked K⁺ depletion in the AngII-infused rats, which is not evident in the control rats.

In AngII-infused rats fed a K⁺-rich meal (A+2K), plasma [K⁺] rose to 4.7±0.1 mmol/L. In a previous study

Protocol 1. Acute K⁺-Rich Meal
On day 13, at 3:00 pm, rats were placed in metabolic cages with water and without food. On day 14, at 7:00 am, overnight urine was collected, and rats were given a gelled meal prepared from powdered chow (TD 88239; Harlan-Teklad, Madison, WI) containing 0.74% NaCl and supplemented to either 2% potassium with KCl (A+2K) or with no potassium added (A+0K and C+40K) as previously described.16 After 3 hours of free access to this food and water, rats were removed and anesthetized for blood and tissue collection. Bladder urine was pooled, with urine collected in metabolic cage before analysis.

Protocol 2. Chronic K⁺-Rich Diet
During the 14 days of AngII infusion, rats were fed either the 2% potassium diet described earlier (A2K) or the same diet with 1% potassium (C1K and A1K, as in control chow). On day 13, at 5:00 pm, rats were placed in metabolic cages with free access to their assigned diets and water for collection of overnight urine. On the morning of day 14, rats were returned to their standard cages with water but without food for 5 hours before they were anesthetized for collection of blood and tissues.

Physiological Measurements
Plasma and urine [Na⁺] and [K⁺] were assessed by flame photometry (Cole-Farmer). BP was measured by tail cuff plethysmography in acclimated rats (Visitech BP2000, Apex, NC).

Transporter Profiling
Kidney homogenates were prepared and assayed as described in detail23 and detailed in Table S1 in the online-only Data Supplement. Signals were analyzed with Odyssey Infrared Imaging System (Li-COR) and software. Arbitrary density units were normalized to mean intensity of control group, defined as 1.0.

Statistical Analyses
Results are presented as individual measurements along with mean±SEM. One-way ANOVA followed by a Tukey’s multiple comparisons post test was used to analyze differences between 3 groups. Statistical tests were calculated using GraphPad Prism (San Diego, CA). A P value of <0.05 was considered significant.

Results
AngII Infusion Leads to Potassium Depletion
In a previous study in male rats, we established that a 3-hour 2%K meal raised plasma [K⁺] from 4.0 to 5.2 mmol/L and increased urine K⁺ excretion (UKV) >10-fold versus rats fed a 3-hour 0%K meal; additionally, NCCp abundance decreased 50%, consistent with a response to shift Na⁺ downstream to cortical collecting duct ENaC, where Na⁺ reabsorption drives K⁺ excretion.16 Our first aim was to determine whether this same 2%K meal protocol would raise plasma [K⁺] and depress NCCp abundance elevated during AngII infusion. Rats infused with AngII (400 ng/kg per minute,14 days) were fasted overnight and then fed a 0%K meal (A+0K) or 2%K meal (A+2K), and non–AngII infused rats were fed the 0%K meal (C+0K). A+0K, or 2K, and C+0K groups consumed 14±2, 10±1, and 14±1 g/3 hours, respectively. Unexpectedly, plasma [K⁺] was significantly lower in A+0K versus C+0K-fed rats (3.6±0.2 versus 4.5±0.1 mmol/L; Figure 1). The protocol was performed twice (and data pooled) with the same lower plasma K⁺ detected in A+0K versus C+0K. This decrease was not detected in our previous studies of AngII-infused rats,4 likely because those rats were fed ad libitum the night before plasma was collected, preserving K⁺ intake, whereas in this series, rats were fasted overnight during which they lost more K⁺ than they took in (K⁺output<K⁺intake). Thus, the overnight potassium deprivation unmasked K⁺ depletion in the AngII-infused rats, which is not evident in the control rats.

In AngII-infused rats fed a K⁺-rich meal (A+2K), plasma [K⁺] rose to 4.7±0.1 mmol/L. In a previous study
using an identical protocol in control rats, plasma [K+] rose to 5.2±0.2 mmol/L. UKV, collected in metabolic cages during the meal and pooled with bladder urine, was measured and means±SEM; *P<0.05 vs C+0K; #P<0.05 vs A+0K after correction for multiple comparisons. Ang II raises plasma [K+] but does not prompt the kaliuresis observed in AngII-infused rats. Figure S3 summarizes abundance of Na-H exchanger, NKCC2, and ROMK. NKCC2 was stimulated by AngII, as reported before; Na-H exchanger, NKCC2, NKCC2-P, and ROMK were unaltered by the K-rich meal. Urinary albumin, a marker of renal injury, detected by Coomassie staining gels, was elevated in all the AngII-infused rats.

Doubling Dietary Potassium During AngII Infusion Prevents NCC Stimulation

The second aim tested the hypothesis that doubling dietary K+ intake would normalize potassium homeostasis during AngII infusion, obviating the drive to increase NCC and NCCp abundance, allowing Na+ delivery to ENaC to drive K+ excretion as in non–AngII-infused rats. A preliminary study in control Sprague–Dawley rats (not infused with AngII) established that chronically doubling chow K+ content from 1% to 2% doubled UKV but did not significantly increase urinary Na+ excretion, plasma [K+], or aldosterone (Table S4), nor did 2%K chow significantly lower NCC or NCCp abundance (Figure S4). Figure 3 and Table S5 compare the physiological responses in AngII-infused rats with doubled K+ intake (A2K) to responses in control (C1K) and AngII-infused (A1K) rats fed 1%K chow. Plasma [K+] was similar in C1K and A1K groups, indicating that K+ depletion is not unmasked in A1K rats fed ad libitum the night before plasma collection, and tended to increase in A2K versus A1K, although insignificantly. In A2K versus A1K, UKV (not urinary Na+ excretion) was doubled, confirming similar dietary intake. Compared with the C1K rats, plasma aldosterone increased 10-fold in A1K rats, reflecting AngII stimulation, and increased 20-fold in A2K rats, reflecting AngII plus potassium stimulation of aldosterone synthesis. As reported and discussed previously, urinary Na+, K+, and volume are higher and plasma [Na+]...
lower in the AngII-infused rats, reflecting increased food intake and thirst.

BP and heart weight were elevated in A1K versus C1K, as expected. Doubling dietary K⁺ (in A2K versus A1K) did not significantly lower systolic BP or reduce cardiac hypertrophy, likely because of the persistent overstimulation of ENaC by AngII.22

Immunoblots of renal cortex homogenates from C1K, A1K, and A2K are summarized in Figure 4 and Figures S5 and S6. As predicted by our hypothesis, doubling K⁺ intake prevented the increase in NCC, NCCpT53, and NCCpS71 abundance levels during AngII infusion: levels in A2K were significantly less than the levels in A1K and not significantly more than baseline levels in uninfused C1K group; both NCC total and NCC phosphorylation were suppressed ≈40% in A2K versus A1K, yet ratio of NCCpT53/total NCC remained elevated in A2K, suggesting that the pool of total NCC was suppressed greater than the pool of NCCpT53 (Table S3). In A1K, levels of the NCC-phosphorylating kinase SPAK and SPAKp were significantly elevated 1.7- and 1.5-fold, respectively, consistent with activation during K⁺ depletion. In A2K, SPAKp (1.2±0.1) did not increase above the levels in C1K, but abundance was not significantly lower than the levels in A1K (1.5±0.1). The results are consistent with the interpretation that SPAKp increases during AngII infusion because of the accompanying K⁺ depletion23,28,29 and is not activated during AngII infusion when K⁺ depletion is prevented by increasing K⁺ intake. Nonetheless, the results still provide
evidence for significant AngII stimulation of total SPAK in the K⁺-restored A2K group (1.5±0.1) versus C1K. The AngII-stimulated increases in α-, β-, and γ-ENaC abundance and activation (cleavage), evident in A1K group, were not further stimulated by doubling K⁺ intake, despite the significantly increased plasma aldosterone and urinary K⁺ excretion in the A2K. This finding reflects the unregulated overactivation of ENaC because of the AngII infusion alone, sufficient to drive the 2-fold increase in K⁺ secretion. Figure S6 summarizes the abundance of Na-H exchanger, NKCC2, NKCC2-P, and ROMK, all unchanged by doubling K⁺ intake in A2K compared with A1K. Additionally, the figure shows that renin is similarly suppressed and urinary albumin similarly increased in all the AngII-infused rats.

**Discussion**

By testing the hypothesis that an acute potassium-rich meal would reduce NCC phosphorylation elevated during chronic AngII infusion, we unexpectedly discovered that rats become potassium depleted by AngII infusion, manifest as kaliuresis and lower plasma [K⁺] after an overnight fast and a 0%K⁺ meal. Chronically doubling potassium intake during AngII infusion normalized potassium homeostasis and prevented the rise in NCC and NCCp. The finding illustrates that some phenotypes associated with AngII infusion, including stimulation of NCC, may be secondary to excessive urinary potassium output and prevented by doubling dietary potassium. AngII stimulation of ENaC abundance and cleavage is the likely culprit that drives the unregulated K⁺ secretion and excretion, which secondarily increases NCC abundance and phosphorylation to reduce Na⁺ delivery to ENaC to blunt K⁺ loss.

That ENaC activity is elevated by AngII is supported by many studies. In isolated tubules, AngII directly and acutely increases ENaC: channel translocation to the plasma membrane, channel activation, and Na⁺ transport mediated by AT1R and superoxide generation, all independent of mineralocorticoid action. Chronic AngII infusion (at a dose and time similar to that used in this study) raises ENaC activity and abundance in the plasma membrane far above that observed with physiological stimuli, also independent of mineralocorticoid action, and amiloride treatment reduces the progression of AngII hypertension, suggesting a nonredundant role for AngII activation of ENaC in the hypertension in this model.

Although direct ENaC channel activity was not assessed in this study, we did assess cleavage and abundance of the alpha and gamma subunits. Cleavage is well established to activate channels in vitro and in vivo, evident as greater amiloride-sensitive natriuresis. There are 2 main routes to reach potassium deficiency: reduce K⁺ intake rate below K⁺ output rate or increase K⁺...
excretion above K⁺ intake by increasing the driving force for K⁺ secretion, for example, by activating ENaC activity or increasing tubular flow rate to increase K⁺ secretion via ROMK or BK channels. AngII is reported to inhibit ROMK channel activity during dietary K⁺ deficiency but not in normal K⁺ diet. Thus, in the setting of AngII infusion, we expect that both the driver (ENaC) and the effector of K⁺ secretion (ROMK) are stimulated; the escape is activation of NCC phosphorylation to reduce Na⁺ delivery to ENaC, all occurring in the setting of normal dietary K⁺ intake.

Why was not K⁺ depletion apparent in previous studies of AngII hypertension? Because in our previous studies, controls and AngII-infused rats were fed normal chow ad libitum the night before blood was collected, and plasma [K⁺] was 4.5 mmol/L in both groups. In protocol 1 of the current study, both groups were fasted overnight and then fed a K⁺-free meal before sampling blood, revealing significantly lower plasma [K⁺] in the AngII-infused versus noninfused rats (3.6 versus 4.5 mmol/L K⁺). During the 3 hours 0%K⁺ meal, urinary K⁺ excretion was not different from zero in the control group.
and 3-fold higher in the AngII-infused group, evidence for K⁺ output is lower despite zero K⁺ intake, which likely led to the fall in plasma [K⁺]. Why did not the acute K⁺-rich meal, with accompanying rise in plasma [K⁺], decrease NCC phosphorylation as reported previously?\(^\text{15,16}\) We postulate that in AngII-infused rats, negative K⁺ balance signals (unidentified) are transmitted to the DCT where they override the signals emanating from the meal and the rise in plasma [K⁺], thus maintaining NCC phosphorylation and favoring sodium reabsorption at the DCT rather than at the cortical collecting duct. This notion is supported by the small increase in urinary K⁺ excretion in the A+2K group; in comparison, UKV increased 7-fold in control rats subjected to the same protocol.\(^\text{16}\)

The AngII-infused rats fed 2% K⁺ containing chow chronically had only a tendency to higher plasma [K⁺] (P=0.07, Figure 3; Table S5). Likewise, doubling K⁺ intake in control rats (not infused with AngII) did not increase plasma [K⁺] nor NCC and NCCp abundance which is a linear function of plasma [K⁺] when dietary K⁺ is chronically varied between 0% and 5%. This relationship is also apparent in control versus AngII-infused rats in this study after an overnight fast, but not apparent in the K⁺-rich meal fed AngII group (Protocol 1; Figure SSA), which is consistent with the lack of significant kaliuresis in this group. We predict that if enough K⁺ was consumed by the rat to actually raise plasma [K⁺] significantly, NCCp would decrease, mediated by the mechanisms recently summarized.\(^\text{28}\)

In the rats chronically fed 2% versus 1% K⁺ diet during AngII infusion, there is only a weak trend of lower NCC and NCCp with higher plasma [K⁺], not surprising because there is 40% lower NCC and NCCp and only a borderline change in plasma [K⁺] in the A2K group (Protocol 2; Figure S7B). There are numerous potential reasons for the lack of a clear association between plasma [K⁺] and NCC in AngII-infused rats: although Terker and Ellison changed K⁺ intake to drive NCCp increase 0.5-fold in the absence of increased ENaC (and potentially ROMK)\(^\text{34}\) in the face of normal dietary K⁺ intake provokes the K⁺ deficiency. When provided with acute or chronic K⁺ supplementation, the AngII-infused rats seem to retain most of it, likely to replenish cellular K⁺ stores. Nonetheless, there is a relationship between NCC activation and urinary K⁺ excretion in the AngII-infused rats: no change in either in response to an acute K⁺ meal (despite rise in plasma [K⁺]) and decreased NCC activation and increased K⁺ excretion in response to chronic K⁺ supplementation. As a future direction, analysis of muscle sodium pumps and potassium pools during AngII infusion (with and without K⁺ supplementation) would reveal the extent of the potassium depletion, as well as the mechanisms activated to replenish the cellular K⁺ stores.

A new study from Terker et al\(^\text{36}\) shows that knocking out mineralocorticoid receptors along the nephrin in mice (KS MR−/−) reduces the abundance and activation of both ENaC and NCC accompanied by salt wasting and hyperkalemia. Analogous to the findings in our current study, the deficient NCC expression in KS MR−/− was corrected by normalizing plasma [K⁺], in this setting by restricting dietary K⁺. The findings indicate that the low NCC abundance in KS MR−/− is secondary to hyperkalemia, not directly because of the loss of MR stimulation.

A recent study by van der Lubbe et al\(^\text{21}\) tested the inverse of the issue addressed in this study, specifically, whether the decrease in NCC abundance provoked by chronic high K⁺ diet was preserved in rats subsequently infused with AngII. The results showed that AngII infusion returned NCC levels to control and doubled NCCp abundance, which the authors attributed to AngII stimulation, and also further increased γ-ENaC cleavage. Although plasma potassium and urinary potassium were not measured in this study, an interpretation consistent with the findings of this study is that the AngII-driven ENaC activation (stimulated above that seen during high K⁺ diet alone) provoked excessive K⁺ secretion, driving K⁺ loss and compensatory increase in NCCp.

Low K⁺ diets have been reported to increase SPAK abundance\(^\text{23}\) attributed to lower DCT cell [Cl⁻]\(^\text{10,28}\) and, thus, to contribute to the increase in NCCp. Wade et al\(^\text{37}\) fed SPAK knockout mice a K⁺-deficient diet and found that the NCC regulatory response was blunted but not eliminated, indicating that other kinases may play a role. We found that, like NCC phosphorylation, the regulation of SPAK increased 50% during AngII infusion and was blunted by doubling dietary K⁺ during AngII infusion, but the response was more subtle: SPAKp was reduced to levels not different from baseline, nor different from the A1K group, and total SPAK remained at 50% above baseline. In our previous studies of AngII-infused rats and mice, SPAK increased after only 3 to 4 days of AngII infusion, before there was any significant increase in ENaC subunit cleavage or BP,\(^\text{8,38}\) which suggests that SPAK may be directly stimulated by AngII,\(^\text{39}\) that is, before K⁺ excretion is increased.

Does AngII regulate NCC independent of changes in plasma [K⁺]? A consensus is developing that acute stimulation of NCC by AngII initially occurs independent of increasing phosphorylation. Infusion of anesthetized rats with AngII for 20 to 30 minutes (with coinfusion of an angiotensin-converting enzyme inhibitor to block local AngII production and prevent hypertension) drives acute trafficking of NCC in multimeric complexes within the apical membrane without any change in NCCp/NCC ratio.\(^\text{40}\) In cultured kidney epithelial cells, NCC is rapidly trafficked to the cell surface in a phosphorylation-independent manner within minutes of an increase in AngII; then, after an hour, AngII induces SPAK-dependent NCC phosphorylation.\(^\text{41}\) The time course of chronic treatment with AngII provides support for direct activation of NCC phosphorylation by AngII: in 3-day AngII-infused rats, NCC and NCCp increased 0.5-fold in the absence of increased ENaC-activating cleavage, urinary K⁺ loss, or K⁺ depletion;\(^\text{42}\) as mentioned earlier, in 4-day AngII-infused mice, SPAK and SPAKp increased significantly before NCC or NCCp increases in the same samples, suggesting that SPAK regulation precedes NCC regulation by AngII; a key caveat is that potassium balance was not measured.

Why does not K⁺ supplementation lower AngII-induced hypertension? Recent population studies show a clear association between higher dietary K⁺ and lower BP.\(^\text{13,14}\) In 2 recent studies, mice fed K⁺-deficient sodium replete diets exhibited elevated BPs.\(^\text{28,29}\) In contrast, in this study, BP had only a tendency to decrease despite pronounced blunting of NCC activation by dietary K⁺ supplementation. The simple explanation...
for this finding is extreme ENaC activation driving inappropriate Na+ reabsorption during AngII infusion—with or without K+ supplementation—necessitating hypertension to drive pressure natriuresis.5

Perspectives
The AngII hypertensive model has been widely used to understand the influence of the central nervous system, blood vessels, immune cells, and kidneys in BP regulation. Here, we provide evidence that treating male Sprague–Dawley rats with AngII for 14 days provokes a K+-deficient state, likely because of ENaC activation, which drives K+ secretion along the cortical collecting duct. This unexpected effect was unmasked after an overnight fast. We previously showed that raising plasma K+ by intravenous infusion or a single K+-rich meal after an overnight fast. We previously showed that raising plasma K+ by intravenous infusion or a single K+-rich meal to suppress NCC activity. Besides, the well-known role of the renin angiotensin aldosterone system in controlling the activity of sodium transporters, we now illustrate a parallel indirect effect of renin angiotensin aldosterone system activation on renal sodium handling driven by altered potassium status. Further, our data provide additional mechanistic support for the cardiovascular benefits of a K+-rich diet to suppress NCC activity.

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Disclosures
None.

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**Novelty and Significance**

**What Is New?**

- Angiotensin II (AngII) infusion provokes kaliuresis, leading to a K+-deficient state. Acute K+ intake does not decrease Na-Cl cotransporter (NCC) phosphorylation nor significantly increase K+ excretion during AngII infusion, as observed in normokalemic rodents. Rather, the ingested K+ is likely used to restore plasma [K+] and K+ intracellular pools.

- Evidence is presented that the AngII stimulation of NCC is secondary to K+ deficiency driven by epithelial Na+ channel stimulation because doubling dietary K+ during AngII infusion prevents increases in NCC and NCC phosphorylation abundance. The results also indicate that elevation in blood pressure is independent of NCC activation during AngII hypertension, likely because of elevated epithelial Na+ channel activity.

**What Is Relevant?**

- When studying AngII hypertension, animals become K+-rather than AngII- or blood pressure–dependent. If interested in detailing AngII or hypertension-specific effects, investigators are advised to consider assessing K+ homeostasis and feeding rodents a K+ enriched diet during AngII infusion to maintain K+ balance to prevent K+ loss.

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**Summary**

Our results suggest that the accumulation and phosphorylation of NCC measured during chronic AngII infusion hypertension is secondary to potassium deficiency driven by epithelial Na+ channel stimulation.
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Supplemental Methods

Animal Protocols. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Keck School of Medicine of the University of Southern California and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Sprague Dawley rats (225–250 g body weight) obtained from Harlan Laboratories (San Diego, CA) were anesthetized intramuscularly with a mixture of ketamine (40 mg/Kg, Phoenix Pharmaceuticals, St. Joseph, MO) and xylazine (8 mg/Kg, Lloyd laboratories, Shenandoah, IA). Rats received a subcutaneous infusion of Angiotensin II (400 ng.kg\(^{-1}\).min\(^{-1}\); Sigma; Ang II infused groups) via osmotic minipumps (Alzet, model 2002, Cupertino, CA) and control group underwent sham surgery.

Protocol 1. Acute K\(^+\)-rich meal. Angiotensin II infusion was continued for 14 days. During this treatment, rats had free access to drinking water and gelled diet prepared from powdered chow (TD 88239; Harlan-Teklad, Madison, WI) supplemented with 2% KCl w/w (~1%K diet) and 0.74% NaCl w/w, equivalent to that found in standard rat chow. From days 10 to 12, rats were acclimated to metabolic cages (Tecniplast, Italy) with free access to food and water. On day 13 at 3:00 PM, food was removed and rats remained in metabolic cages with free access to water for a 16 hour urine collection. On day 14 at 7:00 AM, all urine collection tubes were replaced with clean containers, and the rats were provided with a meal for 3 hrs during which time urine was collected. Specifically, AngII infused rats were divided into two groups: A\(_{+2K}\) (n=5), was provided with a gelled meal supplemented to 4% KCl w/w (~2%K diet which is double that in standard rat chow) and 0.74% NaCl w/w; A\(_{+0K}\) (n=6) was provided with a gelled meal with no KCl added (~0%K) and 0.74% NaCl w/w. Group C\(_{+0K}\) (n=6) was not infused with AngII, and received the 0%K gelled meal. After 3 hr of free access to diet and water, rats were removed from the metabolic cage (at 10:00 AM), and anesthetized with ketamine-xylazine, as described above, for blood and tissue collection. Bladder urine was collected and pooled with 3 hr metabolic cage urine. A C\(_{+2K}\) group was not included because it was already compared to C+0K in a recent study under the same conditions.\(^1\) For reference, in C+2K (n=8) a single 3 hr 2%K meal after an overnight fast increased plasma [K\(^+\)] from 4.0 ± 0.1 to 5.2 ± 0.2 mmol/L; increased urinary [K\(^+\)], [Na\(^+\)], and volume excretion; decreased NCCp by 60%; and marginally reduced cortical Na-K-2Cl cotransporter phosphorylation (NKCC2p) 25%.

Protocol 2. Chronic K\(^+\)-rich diet. In a second set of experiments, rats were divided into three groups: Group A\(_{1K}\) (n=7) and A\(_{2K}\) (n=8) were infused with AngII for 14 days and were provided with the 1%K or the 2%K gelled diets described above, respectively. Group C\(_{1K}\) (n=8) underwent sham surgery and was fed the 1%K diet for the same experimental period.

Blood pressure was measured by tail cuff plethysmography using a Visitech BP2000 system (Visitech Systems Inc., Apex, NC). On days 8, 9, 10 of Ang II treatment, a subgroup of rats was trained for blood pressure measurement (C1K, n=5; A1K, n=3; A2K, n=4; one of the A1K rats quickly expelled the osmotic minipump joining the C1K group.) Each session included 30 blood pressure readings but only the last 20 were considered (displayed in Figure 3). Mean systolic blood pressures data was were
collected and reported for days 11, 12, 13 separately and a mean value was generated for each individual rat from the days 11-13. Simultaneously, from day 10 all animals were acclimated to metabolic cages. On day 13 at 5:00 PM rats were placed in metabolic cages with free access to their assigned diets and water for overnight urine collection (15-18 hr). On day 14, in the morning, rats were returned to their standard cages with water and without food for 5 hours before they were anesthetized, as described in Protocol 1, for blood and tissue collection.

Protocol 3. Chronic K⁺-rich diet without AngII infusion. To assess the impact of chronic 2%K diet on NCC and NCCp abundance, male Sprague Dawley rats (250-290 g body weight, Harlan Laboratories) were divided into three groups and fed either 0%K, 1%K or 2%K diets prepared as described above (n=4 each) during 6 days. On day 5, at 4:00 PM rats were placed in metabolic cages for overnight (16 hr) urine collection with free access to food and water. In the morning, the food was removed and they were euthanized at noon for blood and tissue collection.

Sample collection. Rats were anesthetized as described above, kidneys removed and processed as described below, blood was collected by cardiac puncture and plasma was prepared and quick frozen in aliquots. Urine was collected from the bladder and combined with that collected in the metabolic cage, urine volumes were measured with graduated pipette, and urine was spun at low speed to remove particulates before freezing in aliquots.

Physiological measurements.

Plasma and urine [Na⁺] and [K⁺] were measured by flame photometry (Cole-Parmer).

Aldosterone. In Figure 3 (and Table S5), plasma aldosterone levels were measured by EIA (Cayman Chemical) and in Table S4, levels were determined by ¹²⁵I radioimmunoassay (Coat-A-Count, TKAL kit; Siemens Healthcare Diagnostics).

Homogenate preparation and quantitative immunoblotting. As described,¹,⁶ kidney cortex and medulla were immediately manually dissected and separately diced and homogenized with an Ultra-Turrax T25 (IKA-Labortechnik) at a low setting for 5 min in the following buffer: 5% sorbitol, 5 mM histidine-imidazole (pH 7.5), 0.5 mM disodium EDTA, with the addition of 0.2 mM phenylmethylsulfonyl fluoride, 9 µg/ml aprotinin and 5 µl/ml of a phosphatase inhibitor cocktail (Sigma P2850); Note: the homogenization buffer composition previously published in Rengarajan et. al.¹ inappropriately listed concentrations of the stock solutions, rather than final concentrations. Homogenates were centrifuged at 2,000 g for removal of debris, the supernatant retained and the pellet rehomogenized and recentrifuged. Both 2,000 g supernatants (= “homogenate”) were pooled, quick frozen in aliquots, and stored at -80°C. Protein concentration was determined by BCA assay (Pierce Thermo, Rockford, IL). Cortical homogenates were denatured in SDS-PAGE sample buffer for 20 min at 60°C.⁷ To verify uniform protein concentration and loading, 5 µg of protein from each sample was resolved by SDS-PAGE, stained with Coomassie blue, and multiple random bands quantified and
determined to be uniform, as described⁸ (if not, protein reassessed and gel rerun. Figure S1).

Antibodies used in this study, vendors and dilutions are catalogued in Table S1. Blots were never stripped and reprobed. Samples were analyzed at 1 and ½ volumes on the same blot to verify linearity of the detection system as illustrated for a few lanes of control baseline samples in Figure S2 (1 and ½ amounts specified in Table S1). Signals were detected with Odyssey Infrared Imaging System (Li-COR) and quantified by accompanying software. Arbitrary density units collected were normalized to mean intensity of control group, defined as 1.0. Since the samples were run twice (at 1 and ½ amounts), the normalized values were averaged and mean values compiled for statistical analysis. Only the greater (1X) amounts are shown in figures because the bands are darker for presentation.

**Statistical Analyses.** Data are presented as individual measurements along with mean ± SEM. One-way ANOVA followed by a Tukey’s multiple comparison post-test was used to analyze differences between groups. Statistical tests were calculated using GraphPad Prism 6.0 (San Diego, CA). Differences were regarded significant at \( p < 0.05 \).

**References**


### Table S1. Immunoblot protocol and antibody details

<table>
<thead>
<tr>
<th>Antibody Target</th>
<th>~kDa</th>
<th>Protein per lane (µg)</th>
<th>Primary antibody supplier</th>
<th>Ab host</th>
<th>Dilution</th>
<th>Time</th>
<th>Secondary antibody supplier</th>
<th>Host and target</th>
<th>Dilution</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENaC-α: Full length Cleaved</td>
<td>~100 ~30</td>
<td>80, 40</td>
<td>Loffing (Zurich)</td>
<td>Rb</td>
<td>1:5000</td>
<td>O/N</td>
<td>Invitrogen</td>
<td>GAR 680</td>
<td>1:5000</td>
<td>1 hr</td>
</tr>
<tr>
<td>ENaC-β</td>
<td>~100</td>
<td>60, 30</td>
<td>Loffing (Zurich)</td>
<td>Rb</td>
<td>1:1500</td>
<td>O/N</td>
<td>Invitrogen</td>
<td>GAR 680</td>
<td>1:5000</td>
<td>1 hr</td>
</tr>
<tr>
<td>ENaC-γ: Full length Cleaved</td>
<td>~80 ~60</td>
<td>60, 30</td>
<td>Palmer (Cornell)</td>
<td>Rb</td>
<td>1:1000</td>
<td>O/N</td>
<td>Invitrogen</td>
<td>GAR 680</td>
<td>1:5000</td>
<td>1 hr</td>
</tr>
<tr>
<td>NCC</td>
<td>150</td>
<td>60, 30</td>
<td>McDonough</td>
<td>Rb</td>
<td>1:5000</td>
<td>O/N</td>
<td>Invitrogen</td>
<td>GAR 680</td>
<td>1:5000</td>
<td>1 hr</td>
</tr>
<tr>
<td>NCCpT53</td>
<td>150</td>
<td>60, 30</td>
<td>Loffing (Zurich)</td>
<td>Rb</td>
<td>1:5000</td>
<td>O/N</td>
<td>Invitrogen</td>
<td>GAR 680</td>
<td>1:5000</td>
<td>1 hr</td>
</tr>
<tr>
<td>NCCpS71</td>
<td>150</td>
<td>20, 10</td>
<td>Loffing (Zurich)</td>
<td>Rb</td>
<td>1:5000</td>
<td>2 hrs</td>
<td>Invitrogen</td>
<td>GAR 680</td>
<td>1:5000</td>
<td>1 hr</td>
</tr>
<tr>
<td>NHE3</td>
<td>85</td>
<td>15, 7.5</td>
<td>McDonough</td>
<td>Rb</td>
<td>1:2000</td>
<td>O/N</td>
<td>Invitrogen</td>
<td>GAR 680</td>
<td>1:5000</td>
<td>1 hr</td>
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<tr>
<td>NKCC2</td>
<td>160</td>
<td>15, 7.5</td>
<td>C. Lytle (UCR)</td>
<td>Mu</td>
<td>1:6000</td>
<td>O/N</td>
<td>LiCor</td>
<td>GAM 800</td>
<td>1:5000</td>
<td>1 hr</td>
</tr>
<tr>
<td>NKCC2-pT96T101</td>
<td>160</td>
<td>15, 7.5</td>
<td>Forbush (Yale)</td>
<td>Rb</td>
<td>1:2000</td>
<td>2 hrs</td>
<td>Invitrogen</td>
<td>GAR 680</td>
<td>1:5000</td>
<td>1 hr</td>
</tr>
<tr>
<td>ROMK</td>
<td>~50 ~40</td>
<td>60, 30</td>
<td>Alomone (2009)</td>
<td>Rb</td>
<td>1:1000</td>
<td>O/N</td>
<td>Invitrogen</td>
<td>GAR 680</td>
<td>1:5000</td>
<td>1 hr</td>
</tr>
<tr>
<td>SPAK: Full length SPAK2 KS-SPAK</td>
<td>70 ~65 ~60</td>
<td>20, 10</td>
<td>Delpire (Vanderbilt)</td>
<td>Rb</td>
<td>1:2000</td>
<td>2 hrs</td>
<td>Invitrogen</td>
<td>GAR 680</td>
<td>1:5000</td>
<td>1 hr</td>
</tr>
<tr>
<td>SPAK-pS373</td>
<td>70</td>
<td>80, 40</td>
<td>DSTT, Dundee</td>
<td>Sh</td>
<td>1:4000</td>
<td>2 hrs</td>
<td>Invitrogen</td>
<td>DAS 680</td>
<td>1:5000</td>
<td>1 hr</td>
</tr>
</tbody>
</table>

Protein per lane: to ensure linearity of the detection system, 1 and 1/2 amounts of each sample were assessed. Data using the higher amount is shown in all the figures, although both amounts are used to calculate the changes in abundance, as described in Methods. We refer to detection of NKCC2 rather than NKCC because we have verified that this antibody detects only apically expressed thick ascending limb NKCC2 by immunohistochemistry. Ab = antibody, Mu = mouse, Rb = rabbit, Sh = sheep, O/N = overnight, GAR = goat anti-rabbit, GAM = goat anti-mouse, DAS = donkey anti-sheep.
<table>
<thead>
<tr>
<th>Calculated ratio</th>
<th>C+0K</th>
<th>A+0K</th>
<th>A+2K</th>
</tr>
</thead>
<tbody>
<tr>
<td>NKCC2pT96T101/NKCC2 total</td>
<td>1.0 ± 0.1</td>
<td>0.74 ± 0.04*</td>
<td>0.7 ± 0.1*</td>
</tr>
<tr>
<td>NCCpT53/NCC total</td>
<td>1.0 ± 0.1</td>
<td>1.5 ± 0.2</td>
<td>1.9 ± 0.4</td>
</tr>
<tr>
<td>NCCpS71/NCC total</td>
<td>1.0 ± 0.1</td>
<td>1.5 ± 0.1*</td>
<td>1.6 ± 0.1*</td>
</tr>
<tr>
<td>NCCpS89/NCC total</td>
<td>1.0 ± 0.1</td>
<td>2.3 ± 0.2*</td>
<td>2.6 ± 0.5*</td>
</tr>
<tr>
<td>SPAKpS373/SPAK total</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>1.1 ± 0.1</td>
</tr>
</tbody>
</table>

Table S2. Ratios of phosphorylated NKCC2, NCC and SPAK to total NKCC2, NCC and SPAK, respectively, calculated subsequent to 3 hr K⁺-rich or K⁺ deficient meal. Ratios were calculated from normalized densities of identical samples shown in Figure 2A, assayed in tandem and are expressed as means ± SE. *=P<0.05 vs. C+0K group.

<table>
<thead>
<tr>
<th>Calculated ratio</th>
<th>C1K</th>
<th>A1K</th>
<th>A2K</th>
</tr>
</thead>
<tbody>
<tr>
<td>NKCC2pT96T101/NKCC2 total</td>
<td>1.0 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>NCCpS71/NCC total</td>
<td>1.0 ± 0.1</td>
<td>1.9 ± 0.3*</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>NCCpT53/NCC total</td>
<td>1.0 ± 0.1</td>
<td>1.8 ± 0.3*</td>
<td>1.8 ± 0.3*</td>
</tr>
<tr>
<td>SPAKpS373/SPAK total</td>
<td>1.0 ± 0.1</td>
<td>1.8 ± 0.1</td>
<td>1.7 ± 0.1</td>
</tr>
</tbody>
</table>

Table S3. Changes in ratios of phosphorylated NKCC2, NCC and SPAK to total NKCC2, NCC and SPAK, respectively, in rats fed 1%K vs. 2%K containing chow during AngII infusion. Ratios were calculated from normalized densities of identical samples shown in Figure 4A, assayed in tandem, and expressed as means ± SE. *=P<0.05 vs. C1K group.
<table>
<thead>
<tr>
<th></th>
<th>1%K diet</th>
<th>2%K diet</th>
<th>0%K diet</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PLASMA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>([Na^+]) (mmol/L)</td>
<td>146 ± 4</td>
<td>143 ± 5</td>
<td>137 ± 9</td>
</tr>
<tr>
<td>([K^+]) (mmol/L)</td>
<td>3.8 ± 0.2</td>
<td>3.8 ± 0.5</td>
<td>2.4 ± 0.2*</td>
</tr>
<tr>
<td>Aldosterone (pg/ml)</td>
<td>417 ± 19</td>
<td>471 ± 17</td>
<td>172 ± 17*</td>
</tr>
<tr>
<td><strong>URINE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vol (ml/16 hr)</td>
<td>16 ± 2</td>
<td>26.5 ± 3.8*</td>
<td>25 ± 3.5*</td>
</tr>
<tr>
<td>([Na^+]) (mmol/hr*Kg bw)</td>
<td>0.77 ± 0.07</td>
<td>0.84 ± 0.08</td>
<td>0.76 ± 0.06</td>
</tr>
<tr>
<td>([K^+]) (mmol/hr*Kg bw)</td>
<td>0.67 ± 0.08</td>
<td>1.24 ± 0.12*</td>
<td>0.03 ± 0.002*</td>
</tr>
</tbody>
</table>

**Table S4.** Effects of chronic K⁺-rich diet without AngII infusion on plasma and urine electrolytes. Doubling dietary K⁺ for 6 days did not alter plasma \([Na^+]\), \([K^+]\) or [aldosterone], but increased urine volume and doubled UKV. 0%K diet reduced plasma \([K^+]\), plasma aldosterone and UKV and increased urine volume. Means ± SEM. ★=P<0.05 vs.1%K group.
### Table S5. Physiological parameters measured in response to doubling dietary potassium during AngII hypertension.

Urine samples were collected over a 15-18 hr period in metabolic cages. UV = urine volume; UNaV = urinary Na\(^+\) excretion; UKV = urinary K\(^+\) excretion, CLi = Lithium clearance, SBP = systolic blood pressure. Values represent means ± SEM. ★ = \(P<0.05\) vs. C1K, † = \(P<0.05\) vs. A1K after correction for multiple comparisons.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control, sham (n= 4-8)</th>
<th>AngII + 1%K diet (n = 7)</th>
<th>AngII + 2%K diet (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight initial (g)</td>
<td>252 ± 6</td>
<td>242 ± 6</td>
<td>247 ± 5</td>
</tr>
<tr>
<td>Body weight final (g)</td>
<td>298 ± 6</td>
<td>244 ± 7 ★</td>
<td>241 ± 4★</td>
</tr>
<tr>
<td>Heart weight (g/100 g b.w.)</td>
<td>0.33 ± 0.01</td>
<td>0.41 ± 0.01★</td>
<td>0.41 ± 0.01★</td>
</tr>
<tr>
<td>Kidney weight (g/100 g b.w.)</td>
<td>0.41 ± 0.01</td>
<td>0.40 ± 0.01</td>
<td>0.40 ± 0.01</td>
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<tr>
<td>Plasma [Na(^+)] (mmol/L)</td>
<td>134 ± 1</td>
<td>129 ± 1★</td>
<td>130 ± 1</td>
</tr>
<tr>
<td>Plasma [K(^+)] (mmol/L)</td>
<td>4.2 ± 0.1</td>
<td>4.2 ± 0.2</td>
<td>4.6 ± 0.1</td>
</tr>
<tr>
<td>Plasma aldosterone (pg/ml)</td>
<td>705 ± 72</td>
<td>8133 ± 1661★</td>
<td>16922 ± 3223†</td>
</tr>
<tr>
<td>UV (ml/ hr*Kg)</td>
<td>2.9 ± 0.4</td>
<td>8.9 ± 0.7★</td>
<td>9.7 ± 0.7★</td>
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<tr>
<td>UNaV (mmol/hr*Kg)</td>
<td>0.46 ± 0.02</td>
<td>0.7 ± 0.1★</td>
<td>0.9 ± 0.1★</td>
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<tr>
<td>UKV (mmol/hr*Kg)</td>
<td>0.59 ± 0.02</td>
<td>0.80 ± 0.04★</td>
<td>1.8 ± 0.1★†</td>
</tr>
<tr>
<td>CLi (ml/min*Kg)</td>
<td>0.28 ± 0.02</td>
<td>0.60 ± 0.01★</td>
<td>0.9 ± 0.1★†</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>132 ± 4</td>
<td>197 ± 3★</td>
<td>184 ± 7★</td>
</tr>
</tbody>
</table>
Figure S1. Direct protein staining of the gel was implemented to assess equivalent protein loading per lane. *Top:* Image of a Coomassie stained SDS-PAGE gel (not blot) of renal cortex homogenate samples loaded at 5 µg/lane. *Middle image* indicates the 4 unidentified protein bands arbitrarily chosen for quantitation by the Licor Odyssey system (1 to 4 top to bottom). *Bottom:* Summary display of the density of each of the 4 arbitrary bands (color coded) in each sample as well as the average of the 4 bands (red). The sample to sample variation was less than 5%, viewed as acceptable.
**Figure S2. Linearity of the detection system.** Illustration of the layout of the quantitative immunodetection system: 2 gels of samples loaded at 1X on one gel and ½ X on another gel are placed on one piece of blot matrix for transfer and subsequent antibody incubations and quantitation. Shown are the molecular weight lanes and adjacent baseline control samples; ENaC subunit panels include AngII treated samples from same blots because they have low baseline abundance. The bands illustrated that loading ½ reduced the signal proportionately. See Table S1 for mobility, amounts loaded per lane and antibodies used. In the data figures of the main manuscript the higher (1X) amounts are shown.
Figure S3. A 3 hr K+-rich meal after AngII treatment did not alter the abundance of NHE3, NKCC2 or ROMK in renal cortex. Immunoblots of renal cortex homogenates processed and analyzed as described in Figure 2 and supplemental methods. Density values (normalized to C+0K group =1.0) displayed as mean ± SEM. No differences were detected in transporters between A+0K, A+2K or C+0K. Urinary albumin excretion (by Coomassie stained gel), nearly undetectable in C+0K, was elevated in A+0K and A+2K, as reported previously by many labs.
**Figure S4.** Six-day 2%K diet, *without AngII infusion*, does not alter renal NCC or NCCp abundance. Doubling dietary potassium for 6 days (2%K) diet did not decrease renal cortical homogenate NCC or NCCpT58 abundance, while 0%K diet increased NCC abundance and phosphorylation as reported. Means ± SEM. ★ = P<0.05 vs. 1%K; # = P<0.05 vs. 2%K.

<table>
<thead>
<tr>
<th></th>
<th>1%K diet</th>
<th>2%K diet</th>
<th>0%K diet</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NCC</strong></td>
<td>1.0 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>1.4 ± 0.2*</td>
</tr>
<tr>
<td><strong>NCCpT58</strong></td>
<td>1.0 ± 0.2</td>
<td>1.1 ± 0.2</td>
<td>1.6 ± 0.4*#</td>
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</table>
Figure S5. Doubling potassium intake prevents NCC activation during AngII hypertension. Means ± S.E.M. * =P<0.05 vs. C1K group, # = P<0.05 for A2K vs. A1K after correction for multiple comparisons. NS indicates not significant. When dietary K+ was doubled (A2K), Ang II infusion did not activate NCC, NCCpS71 nor NCCpT53 (0.91 ± 0.04, 1.3 ± 0.1 and 1.6 ± 0.2-fold versus C1K, respectively). ENaC subunit abundance and cleavage increased 1.5 to 3-fold in both A1K and A2K groups.
Figure S6. Abundance of NHE3, NKCC2, NKCC2-P and ROMK were unchanged by doubling K⁺ intake during AngII infusion. Immunoblots of renal cortex homogenates processed and analyzed as described in Figure 2. Density values, normalized to C1K group = 1.0, displayed as means ± SEM. Note that renin is similarly suppressed, and urinary albumin (Coomassie stained gel) similarly increased in both AngII infused groups.
Figure S7. Relationship between NCC, NCCp and plasma [K⁺]

A. Effects of one K⁺-rich meal on NCC and NCCpT53 after 14 days of AngII infusion, overnight fast and one meal: controls fed 0%K (C+0K), AngII infused fed 0%K (A+0K), and AngII infused fed 2%K meal (A+2K). In A+0K, plasma [K⁺] decreases ~1mmol/L together with a 2- and 3-fold increase in NCC and NCCpT53, respectively compared to C+0K. The 3 hr K⁺-rich meal (A+2K) does not significantly reduce NCC nor NCCpT53 despite the 1 mmol/L higher plasma [K⁺], consistent with the idea that NCCp is elevated to limit Na⁺ delivery to ENaC to limit the driving force for K⁺ secretion and loss during AngII treatment.

B. Effects of dietary K⁺ supplementation during 14 day AngII infusion on NCC and NCCpT53. Doubling dietary K⁺ (A2K) prevents NCC activation during AngII infusion, thus, stimulation of NCC appears secondary to K⁺ deficiency.