Kidney360

In Primary Aldosteronism Acute Potassium Chloride Supplementation Suppresses Abundance and Phosphorylation of the Sodium-Chloride Cotransporter

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Key Points

- Potassium chloride intake induced a reduction in sodium-chloride cotransporter (NCC) and phosphorylated NCC in urinary extracellular vesicles from patients with primary aldosteronism during a significantly raised level of endogenous aldosterone.
- Low plasma potassium (secondary to aldosterone excess) may dominate in terms of NCC regulation in the setting of primary aldosteronism.

Abstract

Background Elevated abundance of sodium-chloride cotransporter (NCC) and phosphorylated NCC (pNCC) are potential markers of primary aldosteronism (PA), but these effects may be driven by hypokalemia.

Methods We measured plasma potassium in patients with PA. If potassium was <4.0 mmol/L, patients were given sufficient oral potassium chloride (KCl) over 24 hours to achieve as close to 4.0 mmol/L as possible. Clinical chemistries were assessed, and urinary extracellular vesicles (uEVs) were examined to investigate effects on NCC.

Results Among 21 patients with PA who received a median total dose of 6.0 g (2.4–16.8 g) of KCl, increases were observed in plasma potassium (from 3.4 to 4.0 mmol/L; $P<0.001$), aldosterone (from 305 to 558 pmol/L; $P=0.01$), and renin (from 1.2 to 2.5 mIU/L; $P<0.001$), whereas decreases were detected in uEV levels of NCC (median fold change$_{(post/basal)}$ [FC]=0.71 [0.09–1.99]; $P=0.02$), pT60-NCC (FC=0.84 [0.06–1.66]; $P=0.05$), and pT55/60-NCC (FC=0.67 [0.08–2.42]; $P=0.02$). By contrast, in 10 patients with PA who did not receive KCl, there were no apparent changes in plasma potassium, NCC abundance, and phosphorylation status, but increases were observed in plasma aldosterone (from 178 to 418 pmol/L; $P=0.006$) and renin (from 2.0 to 3.0 mU/L; $P=0.009$). Plasma potassium correlated inversely with uEV levels of NCC ($R^2=0.11$; $P=0.01$), pT60-NCC ($R^2=0.11$; $P=0.01$), and pT55/60-NCC ($R^2=0.11$; $P=0.01$).

Conclusions Acute oral KCl loading replenished plasma potassium in patients with PA and suppressed NCC abundance and phosphorylation, despite a significant rise in plasma aldosterone. This supports the view that potassium supplementation in humans with PA overrides the aldosterone stimulatory effect on NCC. The increased plasma aldosterone in patients with PA without KCl supplementation may be due to aldosterone response to posture challenge.

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Introduction

Primary aldosteronism (PA) is a common and potentially curable form of hypertension. Hypokalemia was considered as a mandatory feature of PA until the more recent recognition of the high prevalence of PA among normokalemic hypertensives (1–4). The thiazide-sensitive sodium-chloride (Cl^-) cotransporter (NCC) is the major transporter on the apical surface of epithelial cells for electroneutral Na^+/Cl^- reabsorption in the early distal convoluted tubule (DCT). Aldosterone and its analogs were thought to be major regulators of NCC through the mineralocorticoid receptor (5–9), but whether these effects in humans are primarily driven by mineralocorticoid-induced hypokalemia is unclear.

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Urinary extracellular vesicles (uEVs) are frequently used as a noninvasive source of renal biomarker discovery (10, 11) and are a reliable tool to monitor specific physiologic responses and disease mechanisms (12). In humans, PA is associated with increased abundance of phosphorylated NCC (pNCC) in uEVs (13). Examination of uEVs from patients with PA undergoing 4 days co-administration of fludrocortisone acetate and oral NaCl loading, with oral potassium chloride (KCl) supplements to correct or prevent hypokalemia during testing, provided evidence that NCC is mineralocorticoid sensitive (14). However, recent studies demonstrated potassium (K^+) supplementation reduced the degree of upregulation of NCC abundance induced by mineralocorticoids in the murine kidney and in uEVs from patients with PA (14–16), suggesting that higher plasma K^+ induced by higher KCl intake may counterbalance mineralocorticoid-induced NCC upregulation.

In the Hypertension Unit of Princess Alexandra Hospital, Brisbane, Australia, before undergoing seated saline suppression testing (SSST) as a means of confirming or excluding PA, patients whose plasma K^+ are <4.0 mmol/L are given oral potassium supplements (in the form of KCl) in variable amounts to achieve or maintain normokalaemia during SSST the next day. By taking advantage of the clinical SSST protocol, the current study aimed to utilize uEVs to address the hypothesis that oral KCl supplementation in patients with PA with elevated endogenous aldosterone will suppress NCC abundance and phosphorylation. Concomitantly, we examined the renal outer medullary potassium channel (ROMK) to assess whether altering plasma K^+ in a setting of aldosterone excess affected K^+ excretion through ROMK.

Materials and Methods

Ethical Issues

The clinical procedures of posture responsiveness testing and seated saline testing were performed in the Hypertension Unit of the Princes Alexandra Hospital, Brisbane, Australia. The laboratory investigations were performed in the Endocrine Hypertension Research Centre, The University of Queensland Diamantina Institute, Brisbane, Australia. Ethical approval was granted by the Metro South Human Research Ethics Committee (HREC/18/QPAH/103).

Recruitment

Hypertensive patients with raised plasma aldosterone-to-renin ratios (ARRs) who were admitted for SSST were invited to participate and provided informed written consent. A total of 38 (24 women/14 men) patients were invited, and all agreed to participate. All patients completed the KCl replacement experiment before SSST.

KCl Replacement

At least 4 weeks before admission, medications affecting plasma aldosterone and renin levels were withdrawn and replaced by other antihypertensive medications (*e.g.*, verapamil, prazosin, doxazosin, moxonidine, and/or hydralazine). Patients were admitted to hospital to ensure the dietary (normal hospital diet) and posture requirements were met and to facilitate monitoring of plasma K^+ levels and other parameters. Detailed clinical routine procedures and sampling time points for the current study are listed in Table 1. In brief, before SSST, patients commenced a 24-hour urine collection at home in the morning of the day of admission. The next day (day 0), aldosterone responsiveness to upright posture was determined by measuring plasma aldosterone at 7:00AM after overnight recumbency and again at 10:00AM after 3 hours of upright posture. On the day of SSST (day 1), an infusion of 2 L 0.9% saline over 4 hours was commenced at 8:00AM, at least 2 hours after rising from bed and at least 30 minutes after assuming a seated position.

The baseline (7:00AM) plasma K^+ results were available at 10:00AM on day 0. If plasma K^+ concentration at 7:00AM was <4.0 mmol/L, participants were given sufficient slow-release KCl (Span-K) up to four times a day (Q6H) in an attempt to achieve as close to 4 mmol/L as possible by the next morning (day 1) before SSST.

For the current study, blood collected at 7:00AM during recumbent posture on day 0 were adopted as the baseline blood measurement (basal), and blood collected on day 1 just before commencement of SSST at 8:00AM during seated posture was adopted as the post-test blood measurement.

Urine Collection and uEV Isolation

Two sterilized 200-ml containers were given to the 38 participants for urine collection. Urine collection time points are listed in Table 1. Briefly, 25–200 ml of midstream morning urine was collected after baseline blood collection and before KCl supplementation on day 0 (baseline urine), and at 6:00–7:00AM on day 1 before commencement of SSST (post-test urine). On day 0, 26 participants who were unable to produce enough urine volume (≥25 ml) at 7:00AM collected urine between 7:00AM and 10:00AM but before KCl replacement commenced; two participants (patients 26 and 34) were on low-dose (1.2 g) KCl supplementation before baseline urine collection. Collected urine samples were immediately treated with protease inhibitor cocktail (Roche cOmplete, EDTA-free; Roche, Basel, Switzerland; 1 tablet per 50 ml urine) before aliquoting and freezing at –80°C. uEVs were isolated using progressive ultracentrifugation techniques with dithiothreitol (DTT) treatment as previously described (14). Obtained uEV pellets were re-suspended in 70–110 μl 1× PBS containing 0.1% v/v SDS, followed by on-ice sonicating with approximately 5–10 cycles at 50% power (10 seconds on/off on Bioruptor Pico; Diagenode, Denville, NJ) and centrifugation for 10 minutes at 17,000 *g* to pellet insoluble residues. Total protein concentration of the obtained supernatant was measured by spectrophotometer (NanoDrop Lite; Thermo Fisher Scientific, Waltham, MA).

Sample Measurements

Measurements of blood and 24-hour urine were performed by Pathology Queensland Laboratory at Princess Alexandra Hospital immediately after collection was completed. Plasma aldosterone was determined by liquid chromatography with tandem mass spectrometry (17), direct renin concentration was determined by chemiluminescent immunoassay (18), and plasma cortisol was determined by immunoassay. Spot urine creatinine concentration was measured using a creatinine urinary detection assay kit

Table 1. Detailed clinical procedures and sampling time point for hypertensive patients with raised plasma ARRs who were admitted for SSST

Time Point	Admission Day	Day of Posture Responsiveness Testing (Day 0)	Day of SSST (Day 1)
6:00AM			1. Urine for uEVs was collected after getting up (post-test spot urine and uEV for the current study)
7:00AM	Home 24-hour urine test started	1. Home 24-hour urine test ended 2. Blood was collected after overnight *recumbency* (results were adopted as baseline blood measurements for the current study) 3. Urine for uEVs was collected after bleeding (baseline spot urine and uEV for the current study)	2. Before SSST, blood was collected 30 minutes after assuming a *seated posture* (results were adopted as post-test blood measurements for the current study) 3. SSST commenced at 8:00AM
10:00AM		4. Blood results at 7:00AM were available For the current study, if plasma $[K^+]$ <4.0 mmol/L, participants were given sufficient oral KCl up to Q6h to achieve as close to 4.0 mmol/Las possible by the next morning at 7:00AM 5. Blood was collected after 3 hours of *upright posture*	
12:00PM			SSST completed
1:00PM			Participants discharged
3:00PM	Patients were invited and consented to participate in the study		

At least 4 weeks before admission, medication affected plasma aldosterone or renin levels was replaced by other antihypertension drugs, *e.g.*, verapamil, prazosin, moxonidine, and/or hydralazine. Patients were admitted to hospital to ensure the dietary (hospital normal diet) and posture requirements were met and to facilitate measurement of plasma K^+ levels and other parameters. Oral KCl supplementation: if participants' plasma K^+ level at 7:00AM on day 0 was <4 mmol/L, participants were given sufficient slow-release KCl (Span-K) up to four times per day (Q6h) to achieve as close to 4 mmol/L as possible by the next morning at 7:00AM. ARR, aldosterone-to-renin ratio; SSST, seated saline suppression testing; uEV, urinary extracellular vesicles; KCl, potassium chloride, given as slow-released KCl (Span-K).

(EIACUN; Invitrogen, Waltham, MA) at the Endocrine Hypertension Research Centre. Spot urinary Na^+ and K^+ were measured by Pathology Queensland Chemistry Department.

Immunoblotting

uEVs were treated with 5× Laemmli buffer (1/4, v/v) and incubated at 60°C for 10 minutes before SDS-PAGE. Ten micrograms of each sample were loaded and separated on 4%–15% Criterion TGX Precast Midi Protein gels (5671083 or 56710985 depending on loading volume; Bio-Rad, Hercules, CA) and were transferred to Turbo Polyvinyl Difluoride Midi Membrane (1704157; Bio-Rad) under 2.5 A and 25 V for 7 minutes on the Bio-Rad Turbo transferring system. Each blot was duplicated for proteins with similar size. Blots were then blocked followed by overnight 4°C incubation with the following antibodies: rabbit anti-NCC (1/1000; AB3553; Merck Millipore, Billerica, MA), rabbit anti-T55/60pNCC (1/1000) (19), rabbit anti-T60pNCC (1/2500) (19), rabbit anti-ROMK (1/500) (20), rabbit anti-ALG-2-interacting protein X (ALIX; 1/2000; ABC40; Merck Millipore), rabbit anti-tumor susceptibility gene 101 (TSG101; 1/2000; MASBC649; Merck Millipore), and rabbit anti-tetraspanin CD9 (CD9; 1/1000; ab92726; Abcam, Cambridge, United Kingdom). NCC and pNCCs were measured by the dominant band between 100 and 150 kDa. ROMK was measured by a complex glycosylated band detected between 50 and 55 kDa, core-glycosylated band at 40 kDa, and unglycosylated band at 37 kDa (20). uEV marker proteins ALIX, TSG101, and CD9 were measured by the dominant bands at 96, 45, and 25 kDa, respectively. Horseradish peroxidase conjugated goat antirabbit IgG antibody (12–348; Merck Millipore) was used as the secondary antibody at 1/20,000 and 1/500 dilutions, respectively, for luminol-based enhanced chemiluminescence (1705061; Bio-Rad) before exposure in configuring signal accumulation mode by Bio-Rad ChemiDoc XRS+ Imager with Image Lab software. Images that did not exceed saturation were exported for analyses.

Table 2. Detailed participants' clinical features

Patient No.	Sex	Age (yr)	BMI (kg/m^2)	SBP/ DBP (mm Hg)	eGFR[a]	Plasma [Creatinine] (μmol/L)[a]	Plasma [K^+] (mmol/L)[a]	No. of Anti-HTN Drugs	Anti-HTN Drugs before SSST (per Day)				Total Dose of Span-K (g) before SSST (per Day)	Response to Upright Posture	SSST Dx	Included in Analyses	Group
									Moxo (μg)	Prazo (mg)	Vera (mg)	Hydra (mg)					
1	F	54	27.3	164/92	>90	61	3.0	2	—	—	180	25	12	U	PA	Included	KCl
2	F	64	47.1	170/82	82	68	3.0	3	600	—	240	100	14.4	R	PA	Included	KCl
3	M	25	25.9	145/95	>90	75	2.8	2	—	—	240	25	16.8	U	PA	Included	KCl
4	M	29	47.3	174/110	>90	66	3.1	2	—	—	480	100	10.8	R	PA	Included	KCl
5	M	43	25.9	140/95	>90	69	3.3	3	200	—	240	25	10.8	R	PA	Included	KCl
6	F	70	39	154/69	77	69	3.4	4	600	4	60	100	6	R	PA	Included	KCl
7	F	54	31.1	136/96	>90	59	3.5	2	—	—	240	50	6	R	PA	Included	KCl
8	F	52	25.6	150/80	>90	55	3.9	0	—	—	—	—	0	U	PA	Included	Non-KCl
9	F	59	43.3	155/84	>90	62	3.8	4	600	1.5	240	100	2.4	U	PA	Included	KCl
10	F	39	25.6	115/78	84	77	3.8[b]	0	—	—	—	—	3.6	—	PA	Included	KCl
11	F	45	38.1	164/71	>90	57	3.9	2	400	—	360	—	3.6	R	PA	Included	KCl
12	F	43	20.8	166/88	>90	56	2.9	2	—	—	480	50	6	U	PA	Included	KCl
13	F	32	22.4	140/80	>90	53	2.9	1	—	—	180	—	13.2	U	PA	Included	KCl
14	M	39	47.3	165/80	>90	89	3.3	1	—	—	240	—	9.6	U	LRH	Excluded	—
15	F	54	28.7	146/82	>90	38	3.8	1	—	—	90	—	6	R	PA	Included	KCl
16	F	72	28	130/80	>90	52	3.7	0	—	—	—	—	2.4	R	LRH	Excluded	—
17	F	53	30.9	142/88	>90	63	3.6	0	—	—	—	—	4.8	R	LRH	Excluded	—
18	M	61	38.9	152/98	>90	73	3.0	4	600	4.5	240	200	7.2	R	PA	Included	KCl
19	M	35	35.9	162/100	>90	81	3.0	3	—	5	480	100	9.6	R	PA	Included	KCl
20	F	45	31.4	136/96	>90	63	4.4	0	—	—	—	—	0	R	PA	Included	Non-KCl
21	M	38	31.8	160/110	>90	77	4.0	4	400	6	240	100	0	R	PA	Included	Non-KCl
22	F	34	27.9	140/92	>90	42	3.9	1	—	—	240	—	0	R	LRH	Excluded	—
23	M	63	30.6	149/95	67	103	4.3	1	—	—	240	—	0	R	PA	Included	Non-KCl
24	F	30	29.2	140/78	>90	54	3.4	2	—	—	180	150	4.8	U	PA	Included	KCl
25	M	43	31.3	144/91	>90	79	3.5	2	—	—	480	50	7.2	U	PA	Included	KCl
26	F	31	23.7	128/86	>90	63	3.5	2	—	—	240	25	6	U	PA	Included	KCl
27	F	36	27	123/78	>90	68	3.6	2	—	—	240	25	6	R	PA	Included	KCl
28	F	51	24.9	125/68	>90	59	4.1	2	—	—	240	150	0	U	PA	Included	Non-KCl
29	F	36	32.8	129/72	>90	59	4.0	1	—	—	180	—	0	R	LRH	Excluded	—
30	M	44	28.5	145/95	>90	73	4.1	2	—	—	240	37.5	0	—	PA	Included	Non-KCl
31	F	49	39.9	142/86	>90	68	3.9	2	—	2	180	—	0	U	PA	Included	Non-KCl
32	F	57	26.9	140/102	>90	62	3.1	1	—	—	120	—	0	R	PA	Included	Non-KCl
33	F	63	30	144/84	90	64	3.9	1	—	—	180	—	0	R	PA	Included	Non-KCl
34	M	55	33.7	170/106	>90	61	3.3	4	200	2	240	150	4.8	U	PA	Included	KCl
35	M	52	24.8	116/76	82	93	3.6	1	—	—	240	—	5.4	U	PA	Included	KCl

Table 2. (Continued)

Patient No.	Sex	Age (yr)	BMI (kg/m^2)	SBP/ DBP (mm Hg)	eGFR[a]	Plasma [Creatinine] (μmol/L)[a]	Plasma [K^+] (mmol/L)[a]	No. of Anti-HTN Drugs	Anti-HTN Drugs before SSST (per Day)				Total Dose of Span-K (g) before SSST (per Day)	Response to Upright Posture	SSST Dx	Included in Analyses	Group
									Moxo (μg)	Prazo (mg)	Vera (mg)	Hydra (mg)					
36	F	37	42.4	164/96	>90	74	3.7	4	400	2	240	150	3.6	R	PA	Included	KCl
37	M	39	36.8	170/98	75	108	4.2	4	600	4	360	75	0	R	PA	Included	Non-KCl
38	M	45	48.8	160/100	86	92	4.3	4	400	4	240	25	0	U	LRH	Excluded	—

BMI body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; plasma [K^+], plasma potassium concentration; anti-HTN, antihypertensive; SSST, seated saline suppression testing; Moxo, moxonidine; Prazo, prazosin; Vera, verapamil; Hydralazine; Span-K, slow-release potassium chloride; Dx, diagnosis of primary aldosteronism; F, women; M, men; U, posture-unresponsive; R, posture-responsive; PA, primary aldosteronism; LRH, low renin essential hypertension. KCl, the group of patients who received KCl supplementation; Non-KCl, the group of patients who did not receive KCl.

[a]Bloods for eGFR and plasma creatinine were collected on the day of admission; bloods for plasma K^+ were collected in a recumbent posture at 7:00AM on day 0.

[b]The 7:00AM blood sample was hemolyzed, and the plasma K^+ level at 10:00AM was 3.8 mmol/L. Hence, KCl supplementation was given and commenced at midday.

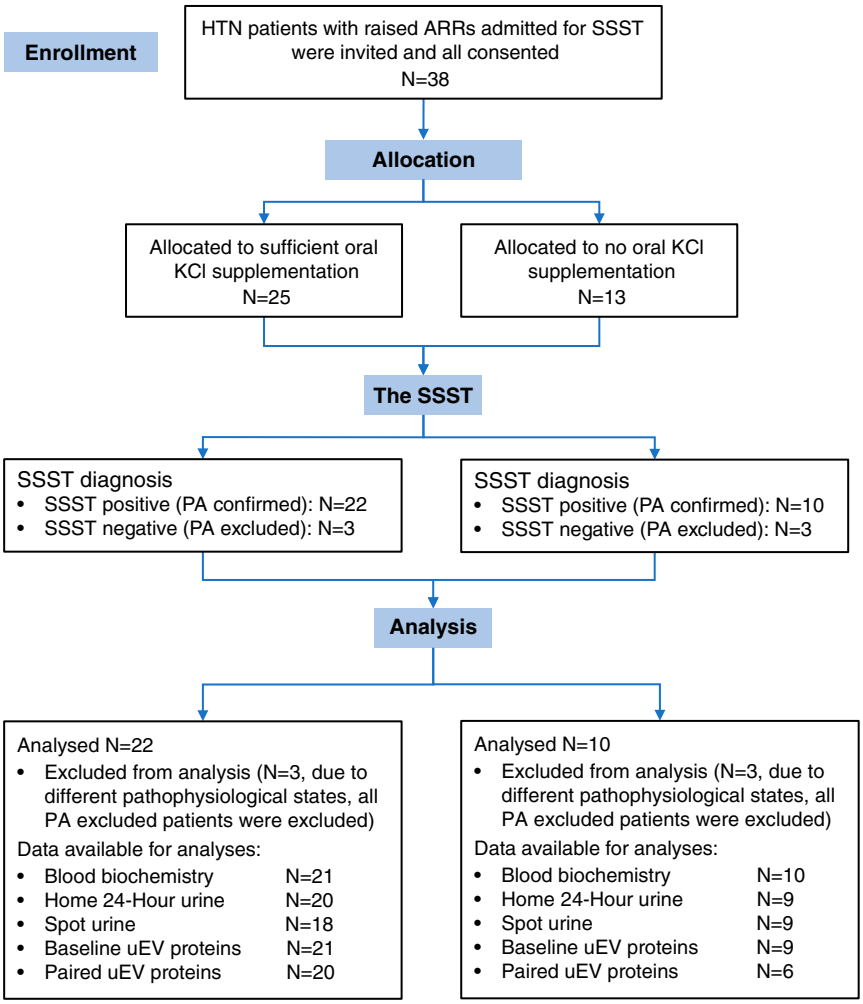

Figure 1. | **Flow diagram of report numbers of participants at each stage of study.** Comparisons of blood biochemical parameters were performed between 21 (13 women/eight men) in the KCl group (excluding patient 10) and ten (six women/four men) in the non-KCl group. Home 24-hour urinary parameters were compared between 20 (12 women/eight men) in the KCl group (excluding patients 7 and 9) and nine (five women/four men) in the non-KCl group (excluding patient 28). Spot urinary parameters were compared between 18 (12 women/six men) in the KCl group (excluding patients 15, 18, and 35) and nine (five women/four men) in the non-KCl group (excluding patient 31). A total of 30 (18 women/12 men) patients with PA were included in the baseline analyses of NCC abundance and phosphorylation, and 26 (16 women/ten men) patients with PA were included in paired comparisons. NCC, sodium-chloride cotransporter; HTN, hypertension; ARRs, aldosterone-to-renin ratios; SSST, seated saline suppression testing; *N*, sample size; KCl, potassium chloride; PA, primary aldosteronism; uEV, urinary extracellular vesicles.

Analyses Inclusion Criteria

Due to the different physiologic states, only patients with confirmed PA were included in all analyses. Immunoblotting detection of at least two EV-enriched proteins (ALIX, TSG101, or CD9) in each sample was considered successful isolation of uEVs, and patients in whom uEVs were successfully isolated from both basal and post-test samples were included in the comparison analyses. Samples included in uEV protein analyses are listed in Supplemental Table 1.

Statistical Analyses

Calculations were processed with R (The R Foundation for Statistical Computing, Vienna, Austria). Due to blood at baseline being hemolyzed, patient 10 was excluded from the comparisons of blood biochemical parameters. Therefore, comparisons of blood biochemical parameters were performed in a total of 37 patients. Because the volume of spot urine collected from patients 15, 18, 29, 31, and 35 remained inadequate after uEV isolation, measurements of creatinine, Na^+, and K^+, the comparisons of spot urinary electrolytes-to-creatinine ratios were performed in a total of 32 patients. Paired Wilcoxon tests were performed to compare the differences of biochemical parameters in blood samples collected at 7:00AM on day 0 and 8:00AM on day 1 and in spot urine electrolytes-to-creatinine (Na^+/creatinine and K^+/creatinine) at basal and post test. In immunoblotting analyses, absolute abundances of analyzed proteins were analyzed with ImageJ (National Institutes of Health, Bethesda, MD). To minimize errors due to alterations in actual uEV biogenesis or excretion rates, relative protein abundances (derived by dividing the absolute abundances of proteins of interest by the sum abundance of ALIX,

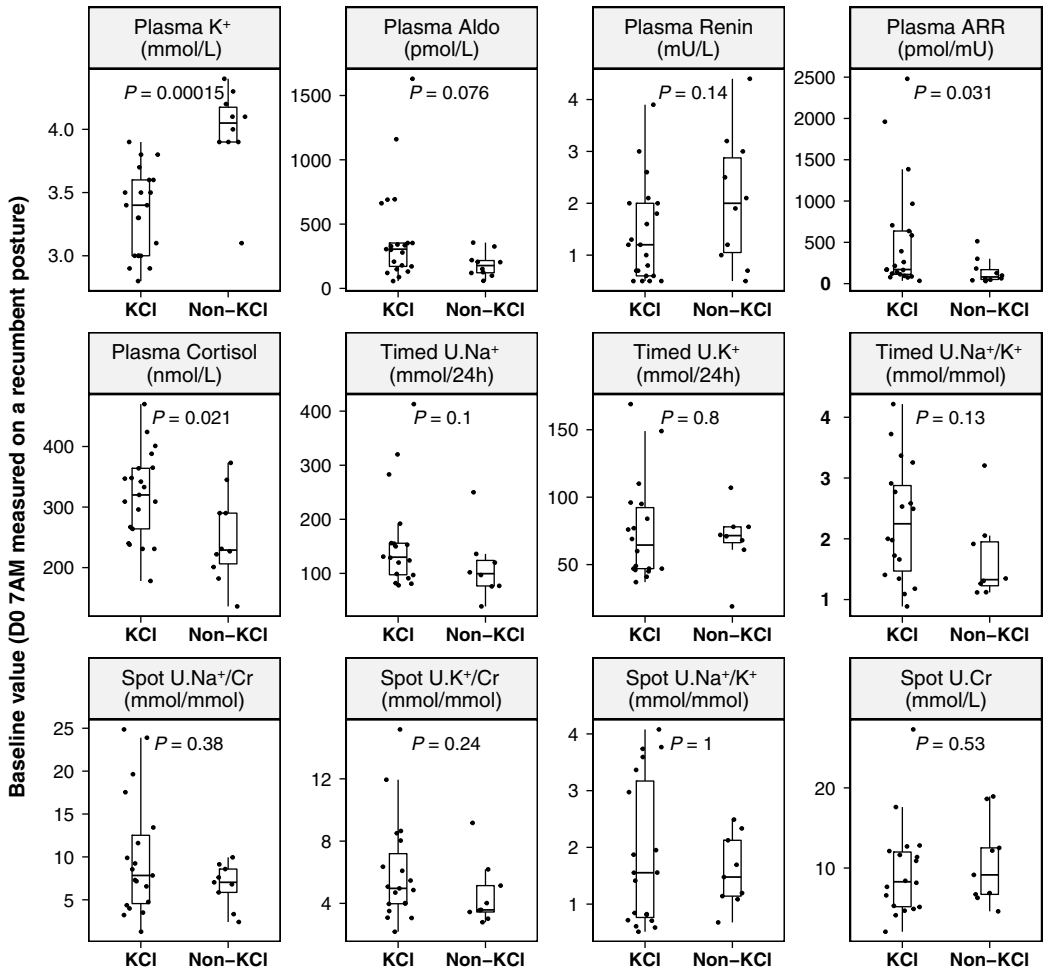

Figure 2. | Baseline (7:00AM) biochemical differences between PA in the KCl group and those in the non-KCl group. KCl, patients with PA who received oral KCl (Span-K) supplementation; non-KCl, patients with PA who did not receive oral KCl supplementation; K^+, potassium; Aldo, aldosterone; Timed U.Na^+/Cr, 24-hour urinary sodium-to-creatinine ratio; Timed U.K^+/Cr, 24-hour urinary potassium-to-creatinine ratio; Timed U.Na^+/K^+, 24-hour urinary sodium-to-potassium ratio. Spot U.Na^+/Cr, spot urinary sodium-to-creatinine ratio; Spot U.K^+/Cr, spot urinary potassium-to-creatinine ratio; Spot U.Na^+/K^+, spot urinary sodium-to-potassium ratio; *P* values on the basis of unpaired Wilcoxon test; median, the median value of each parameter in each group.

TSG101 and CD9 in each patient's sample) were used for comparisons. Wilcoxon tests were performed to assess baseline differences in analyzed proteins. Paired *t* tests were performed to assess the changes in the relative abundances of analyzed proteins (log10 transformed). Pearson's correlations were sought between log10 transformed relative abundances of analyzed proteins and biochemical parameters. If baseline urines for uEV were collected after 10:00AM, 10:00AM biochemical results were used. $P<0.05$ was considered statistically significant. Data are presented as median (range) unless stated otherwise.

Results

Participants' Characteristics

A total of 38 (24 women and 14 men) hypertensive patients with repeatedly raised ARRs were recruited, and all agreed to participate (Table 2). SSST was positive in 32, thereby confirming the diagnosis of PA, and negative in six, in whom PA was excluded (designated low renin hypertension [LRH]). Due to the different physiologic states, only patients with confirmed PA were included in the analyses. The report numbers of participants at each stage of study are depicted in Figure 1. Among the 32 (19 women/12 men) patients with PA, the median age was 45 (25–70) years old, with a median body mass index of 30.3 (20.8–47.5) kg/m^2, median blood pressure of 146 (115–174)/89 (68–110) mm Hg, and were on a median of 2 (0–4) antihypertensive medications without affecting plasma aldosterone and renin levels.

The baseline blood sample from patient 10 was hemolyzed, but her plasma K^+ at 10:00AM on day 0 was 3.8 mmol/L. Hence, oral KCl supplementation was given and commenced at midday on the same day. Of the remaining 31 (18 women/12 men) patients with PA, 21 (13 women/eight men) whose baseline plasma K^+ was <4 mmol/L were given KCl supplementation (hereafter referred to as the KCl group), and ten (six women/four men) did not receive KCl (hereafter referred to as the non-KCl group). Among the ten in the non-KCl group, nine had baseline K^+ levels that were

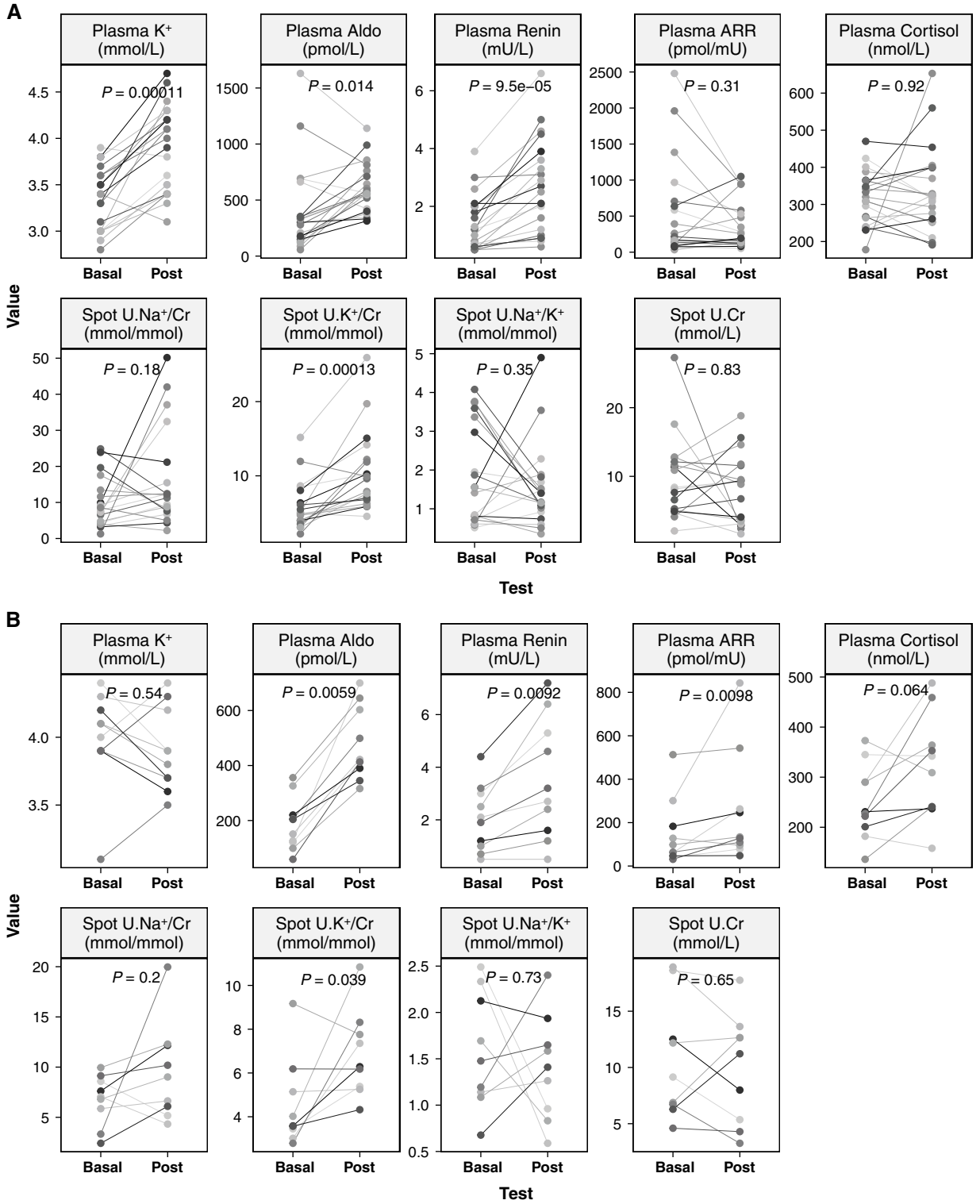

Figure 3. | **Physiological effects of KCl supplementation in PA.** Physiologic changes within the 25-hour experimental period in the KCl group (A) and the non-KCl group (B). Basal, baseline values measured at 7:00AM on day 0 in a recumbent posture; Post, post-test values measured at 8:00AM on day 1 during seated posture; *P* values on the basis of paired Wilcoxon test.

either ≥4 mmol/L (n=6) or only just below 4 mmol/L (n=3), whereas the remaining patient (patient 32) had a baseline K^+ of 3.1 but was not given KCl, despite meeting the protocol criteria.

Baseline Biochemical Differences

Baseline plasma K^+ was significantly higher (P<0.001) in the non-KCl group (4.0 [3.1–4.4] mmol/L) than in the KCl group (3.4 [2.8–3.9] mmol/L; Figure 2), whereas plasma

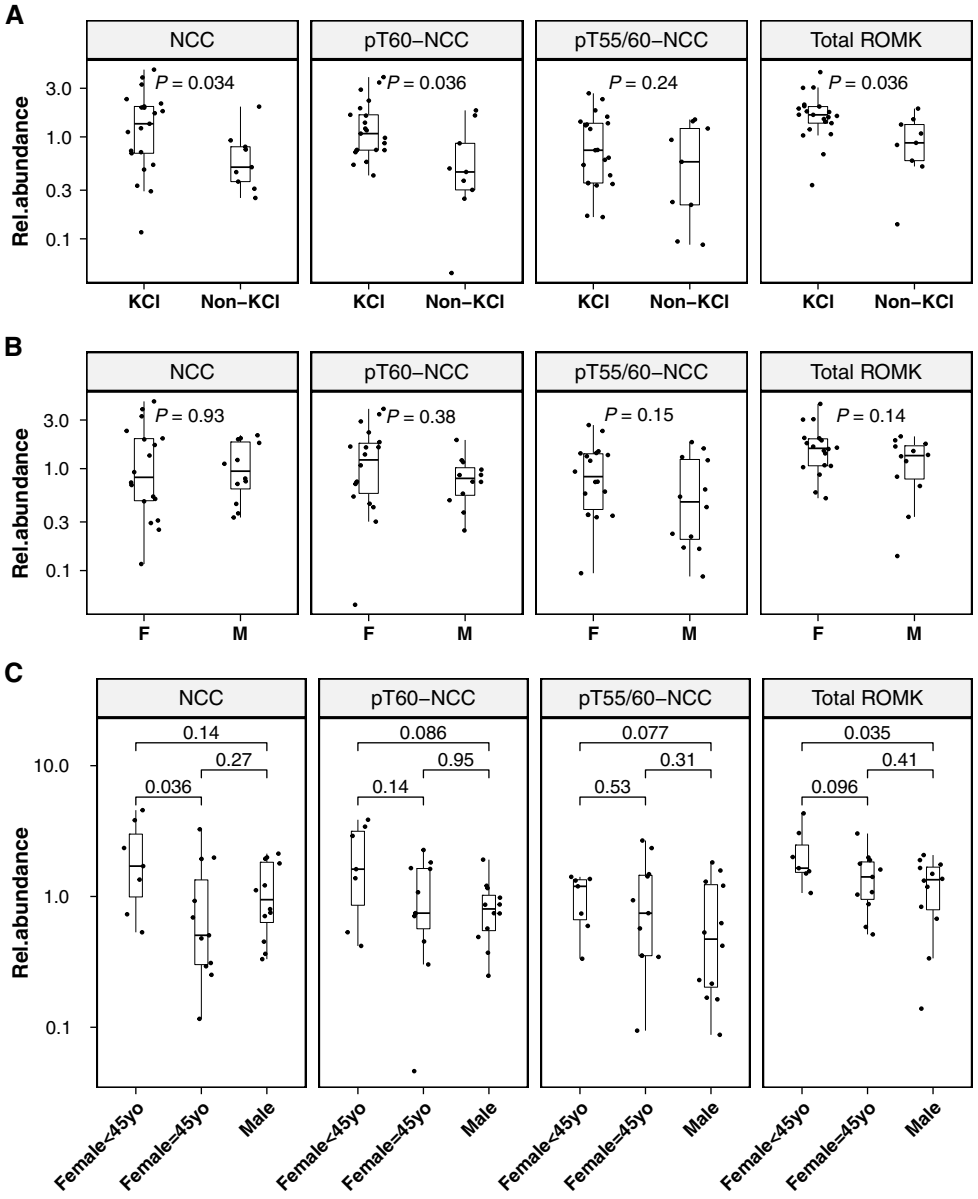

Figure 4. | **Baseline uEV protein differences**. Baseline differences in uEV levels of analyzed proteins in participants with PA according to allocation of oral KCl supplementation (A) and sex (B and C). KCl, participants in the KCl group; non-KCl, participants in the non-KCl group; F, women; M, men; Female<45yo, women who were <45 years old; Female≥45yo, women who were ≥45 years old; Rel.abundance, relative protein abundance (the ratio of protein absolute abundance to the sum abundance of ALIX, TSG101, and CD9); *P* values on the basis of unpaired Wilcoxon test.

levels of aldosterone (178 [59–356] versus 305 [57–1630] pmol/L; $P=0.08$), ARR (81 [31–513] versus 171 [34–2480] pmol/mIU; $P=0.03$), and cortisol (229 [136–373] versus 320 [178–470] nmol/L; $P=0.02$) were relatively lower in the non-KCl group than in the KCl group. There were no statistical differences in 24-hour urinary levels of Na^+, K^+, and Na^+/K^+, and in spot urinary Na^+/creatinine, K^+/creatinine, and ratio of Na^+/K^+ and creatinine between the two groups.

Physiologic Effects of KCl Supplementation in PA

In the KCl group (Figure 3A), plasma K^+ increased ($P<0.001$) from a median of 3.4 (2.8–3.9) to 4 (3.1–4.7) mmol/L in response to a median of 6.0 (2.4–16.8) g total dose of KCl, although plasma K^+ fell in two patients (from 3.9 to 3.8 mmol/L in patient 11, despite 3.6 g KCl, and from 3.4 to 3.1 mmol/L in patient 24, despite 4.8 g KCl). Spot urine K^+/creatinine increased from 5.0 (2.2–15.2) to 9.8 (4.5–25.9) mmol/mmol ($P<0.001$). Increases were also detected in plasma aldosterone (from 305 [57–1630] to 558 [313–1140] pmol/L; $P=0.01$) and direct renin concentration (from 1.2 [0.5–3.9] to 2.5 [0.6–6.6] mIU/L; $P<0.001$). There were no apparent changes in plasma levels of ARR ($P=0.31$) and cortisol ($P=0.92$), spot urine Na^+/creatinine ($P=0.25$), spot urine Na^+/K^+ ($P=0.39$), and spot urine creatinine concentration ($P=0.83$).

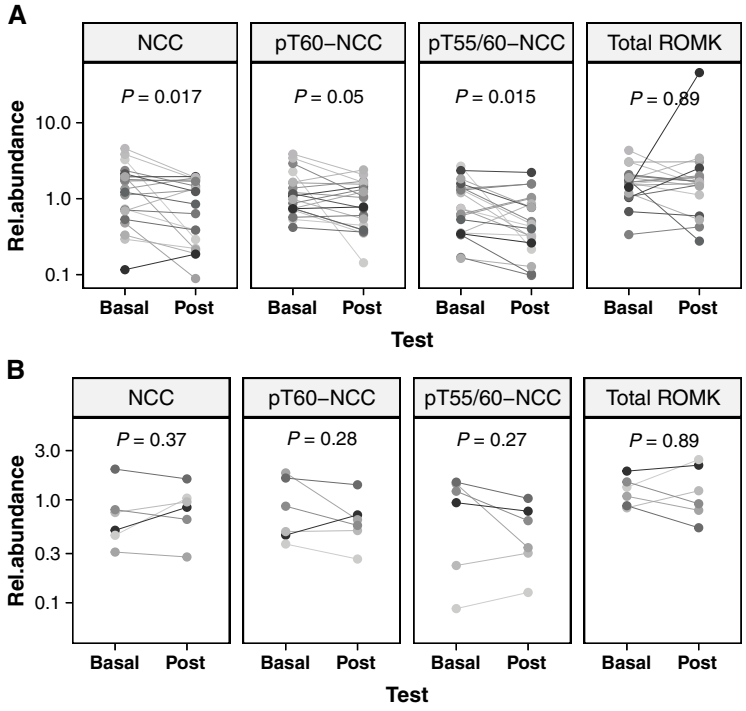

Figure 5. | **Acute KCl supplementation reduces NCC and pNCC in PA**. Changes of relative abundances of analyzed proteins in participants with PA (A) who received and (B) who did not receive oral KCl supplementation. Basal, baseline; Post, post-test; *P* values on the basis of paired *t* test (log10 transformed relative protein abundance was applied).

Among the 10 patients (six women/four men) in the non-KCl group (Figure 3B), there were no apparent changes in plasma K^+ (basal 4.0 [3.1–4.4] versus post 3.8 [3.5–4.4] mmol/L; P=0.54), but increases in the spot urine K^+/creatinine were observed, from 3.6 [2.8–9.2] to 6.3 [4.3–10.8] mmol/mmol (P=0.04). Like those in the KCl group, there were increases in plasma levels of aldosterone (from 178 [59–356] to 418 [316–700] pmol/L; P=0.006) and renin (from 2.0 [0.5–4.4] to 3 [0.5–7.2] mIU/L; P=0.009), and an additional increase in plasma ARR (from 81 [31–513] to 131 [48–843] pmol/mIU; P=0.01). There were no apparent changes in plasma cortisol (P=0.06), spot urine Na^+/creatinine (P=0.2), spot urine Na^+/K^+ (P=0.73), and spot urine creatinine (P=0.65).

uEV Characterization

There was insufficient sample quantity to allow for characterization of the uEVs by transmission electron microscopy and nanoparticle tracking analysis, but we have previously used these approaches to examine uEV pools isolated from humans by the same protocol and seen very clear uEV structures, and the uEVs' size distribution was within the EVs appropriate range (30–1000 nm in diameter) (21, 22). Immunoblotting detection of at least two EV-enriched proteins (ALIX, TSG101, or CD9) in each sample was considered successful isolation of uEVs, and patients in whom uEVs were successfully isolated from both basal and post-test samples were included in the comparison analyses (Supplemental Figure 1, Supplemental Table 1). The observation of a shift in the ALIX bands in multiple samples may be due to ALIX truncation by the endosomal sorting complex required for transport machinery (23) or related to the presence of Tamm Horsfall protein (24). During the test, no apparent changes were observed in spot urine creatinine concentration (Figure 3) and in absolute abundance of the EV-enriched proteins (Supplemental Figure 2), rendering an effect of potassium on uEV concentration less likely (12, 24).

Baseline uEV Protein Differences

Baseline proteins were compared by allocation of oral KCl supplementation and sex (Figure 4). PA in the KCl group (N=21; 13 women/eight men) had higher abundances of NCC (1.35 [0.12–4.57]; P=0.03), pT60-NCC (1.08 [0.42–3.86], P=0.04), and total ROMK (1.65 [0.34–4.32], P=0.04) than PA in the non-KCl group (N=9; five women/four men; NCC: 0.5 [0.25–1.98]; pT60-NCC: 0.45 [0.05–1.82]; total ROMK: 0.87 [0.14–1.9]; Figure 4A). No sex-related differences were detected in analyzed proteins when total women were compared with total men (Figure 4B). By dividing women into two groups on the basis of the age of 45 into "likely premenopausal" and "likely postmenopausal" (25), women who were <45 years old exhibited a significantly higher level of NCC (1.71 [0.53–4.57], P=0.04) compared with women who were ≥45 years of age (0.5 [0.12–3.26]) and a higher level of ROMK (1.65 [1.06–4.32], P=0.04) compared with that in men (1.34 [0.14–2.07]; Figure 4C).

Acute KCl Reduces NCC Abundance and Phosphorylation

In the KCl group, there were significant decreases in the abundances of NCC (median fold change$_{(post/basal)}$

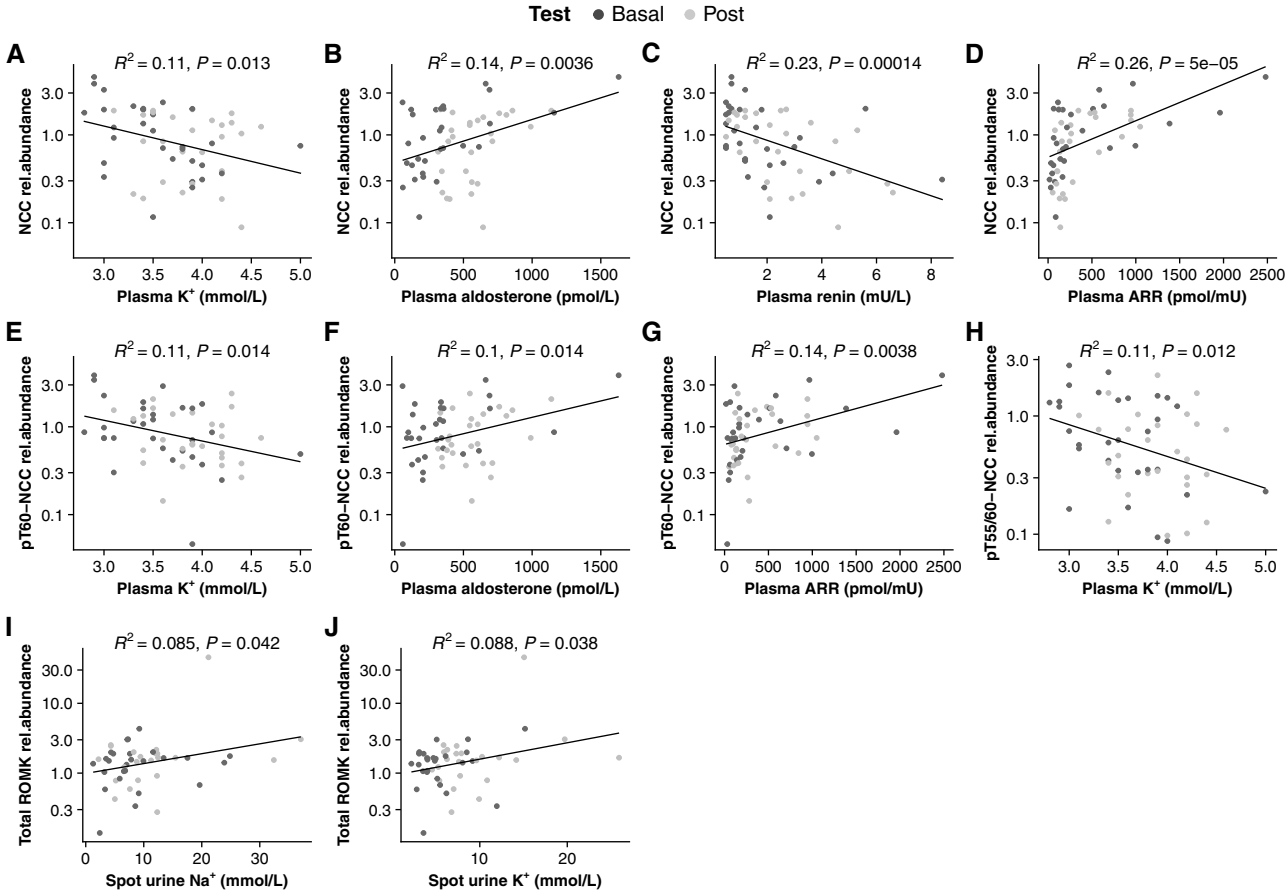

Figure 6. | Notable correlations among all participants with PA during the test. Basal, baseline values (colored in dark gray), biochemical parameter measured at 7:00AM on day 0 in a recumbent posture; Post, post-test values (colored in silver), biochemical parameter measured at 8:00AM on day 1 during a seated posture; rel.ab, relative protein abundance (the ratio of protein absolute abundance to the sum abundance of ALIX, TSG101, and CD9).

[FC]=0.71 [0.09–1.99]; P=0.02), pT60-NCC (FC=0.84 [0.06–1.66]; P=0.05), and pT55/60-NCC (FC=0.67 [0.08–2.42]; P=0.02). By contrast, in the non-KCl group, no significant changes in NCC, pT60-NCC, and pT55/60-NCC were seen. There were no significant changes in uEV levels of ROMK in either the KCl or non-KCl group (Figure 5, Supplemental Figure 2).

K^+ Inversely Correlates with NCC Abundance and Phosphorylation

The uEV levels of NCC (R^2=0.11; P=0.01), pT60-NCC (R^2=0.11; P=0.01), and pT55/60 (R^2=0.11; P=0.01) were negatively correlated with plasma [K^+] among all PA participants during the test (Figure 6, A, E, and H). In addition, NCC positively correlated with plasma aldosterone (R^2=0.14; P=0.004) and ARR (R^2=0.26; P<0.001) and negatively correlated with plasma renin (R^2=0.23; P<0.001; Figure 6, B–D). pT60-NCC positively correlated with plasma aldosterone (R^2=0.1; P=0.01) and ARR (R^2=0.14; P=0.004; Figure 6, F and G). Furthermore, ROMK weakly positively correlated with spot urine Na^+/creatinine (R^2=0.09; P=0.04) and K^+/creatinine (R^2=0.09; P=0.04; Figure 6, I and J). ΔKCl dose strongly negatively correlated with fold change of NCC (R^2=0.3; P=0.004; Figure 7A). There were no significant associations between Δplasma K^+ and fold change of NCC (R^2=0.11; P=0.09) and between Δspot urine K^+/creatinine and fold change of pT55/60-NCC (R^2=0.17; P=0.05; Figure 7, B and C). There were no additional associations of the analyzed proteins with other biochemical parameters (Supplemental Figure 3).

Discussion

Aggressively correcting hypokalemia by 24-hour oral KCl supplementation before SSST increased median plasma [K^+] in the KCl group, whereas in the non-KCl group, plasma [K^+] remained unchanged. However, during the 24-hour period, increases in plasma aldosterone were observed in both KCl (1.8-fold) and non-KCl (2.2-fold) groups, which is likely due to difference in posture between baseline (recumbent) and post-test (upright) sampling as evidenced by the observed rises in direct renin concentration in both groups (26). Upright posture has a significant stimulatory effect on renin beginning at 15 minutes and peaking between 60 and 120 minutes, and aldosterone secretion directly correlates with the elevation in the renin activity during the 120 minutes in an upright posture (27–30). Although there are posture (or angiotensin

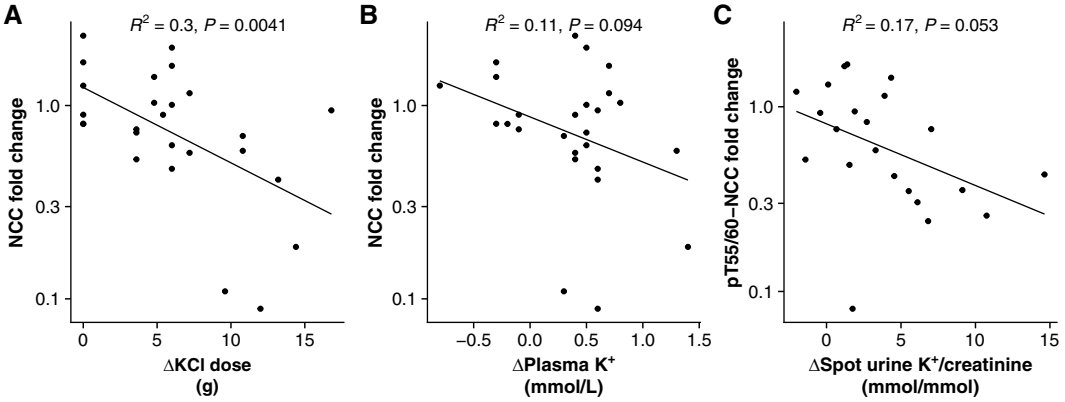

Figure 7. | ΔKCl dose strongly negatively correlated with fold change of NCC. Correlations of ΔKCl dose (A), Δplasma [K^+] (B), and Δspot urine K^+/creatinine (C) with fold changes of NCC abundance and phosphorylation in participants with PA.

II)-unresponsive forms of PA, in which adrenocorticotropic hormone assumes a dominant role over angiotensin II in regulating aldosterone production (31), in the current study, plasma aldosterone levels in most patients in the non-KCl group were responsive to the upright posture (Table 2). Furthermore, blood samples were collected at 7:00AM at baseline and just before 8:00AM post test, and there were no statistically changes in plasma cortisol in either group, making it unlikely that the changes in plasma aldosterone reflected its circadian rhythm. In the KCl group, the rise in plasma K^+ may also have contributed to the rise in plasma aldosterone, but no apparent correlation was detected (Supplemental Figure 4).

By using uEV, we observed a significant suppressive effect of oral KCl supplementation on NCC abundance and phosphorylation (pT55/60-NCC and pT60-NCC) in patients with PA, despite an increase in endogenous aldosterone, whereas no obvious changes were detected in patients with PA who did not receive KCl. uEV analysis is thought to offer only an indirect way to assess NCC abundance in the DCT and might reflect changes in NCC trafficking rather than changes in NCC expression. A recent large-scale unbiased analysis demonstrated that uEV proteins track the abundance of the parent protein in the kidney, thus supporting the use of uEV protein changes to monitor specific physiologic responses and disease mechanisms (12). Moreover, human uEVs reproduced the inverse correlations of plasma K^+ and the positive association of plasma aldosterone with kidney NCC and pT60-NCC that have been observed in *in vivo* studies and clinical studies of NCC in uEVs (14, 21, 32–34). The negative correlation between ΔKCl dose and NCC fold change suggests that at least part of the variance in NCC may be a result of the KCl dose. And this variance has also been observed in two groups of patients with PA who underwent 4-day administration of exogenous mineralocorticoid (14, 15). The negative correlation between ΔKCl dose and NCC fold change, the greater change of spot urine K^+/creatinine in the KCl group, and negative correlations of plasma K^+ with NCC abundance and phosphorylation infer that reduced NCC phosphorylation may be related to promoting kaliuresis to avoid acute hyperkalemia induced by KCl supplementation (35–38).

Interestingly, patients with PA in the non-KCl group exhibited relatively low baseline plasma aldosterone (approximately half of that in PA in the KCl group) and doubled their aldosterone levels post test. However, the significant rise in plasma aldosterone in non-KCl patients with PA did not lead to overall increased NCC abundance and phosphorylation. In contrast, baseline NCC and pT60-NCC were significantly higher in patients with PA in the KCl group than those in the non-KCl group in association with relatively low plasma K^+. These observations support the hypothesis that elevated endogenous aldosterone *per se* does not result in increased NCC abundance and phosphorylation.

In the current study, alteration of plasma K^+ level was achieved by oral KCl supplementation, which leaves a question as to whether plasma K^+ independently suppresses NCC abundance and phosphorylation or whether it acts concurrently with Cl^-. Our recent study demonstrated that significantly increased plasma Cl^- in patients with PA during acute saline infusion appeared only to cause changes in NCC and pNCC due to urine dilution (21). Therefore, it is less likely that plasma Cl^- contributes to reduction in uEV levels of NCC abundance and phosphorylation. The putative renal "K^+ switch" mechanism is a relatively cohesive model that may provide a mechanism linking K^+ intake and NCC regulation (14, 32, 39–41). The "switch" activates NCC in response to low K^+ intake, and "turns off" NCC in response to high K^+ intake. Hence, although aldosterone stimulates ENaC to promote distal Na^+ reabsorption and K^+ excretion in the aldosterone-sensitive distal nephron, the K^+ concentration itself regulates NCC activity to alter the Na^+ amount delivered downstream to ENaC, at least in the conventional model (42, 43).

The current study provides further evidence that K^+ regulates NCC activity during a state of increased endogenous aldosterone in patients with PA. Although other interventions, including adrenalectomy and spironolactone, reduced NCC in adrenal-intact animals (44, 45), and conditional tubule knockdown of the mineralocorticoid receptor also reduces NCC (46), these effects might also be attributable to plasma K^+. Because this was an observational study undertaken opportunistically while patients were admitted

for a clinical procedure (SSST), hospital 24-hour urine measurements at both baseline and completion of the test period (which would have meant delaying SSST and therefore extending the hospital admission for a further 24 hours) were not clinically feasible. We instead used the creatinine normalized spot urine values of Na^+ and K^+ as indicators of changes in distal nephron function. The significant increase in urinary K^+/creatinine accompanied by the trend to an increase of urinary Na^+/creatinine in patients who received oral KCl is in accordance with the phenomenon of K^+-induced natriuresis. However, we were unable to assess if the kaliuresis induced by oral KCl supplementation was ENaC dependent, as reported by others (47), because ENaC (which has low abundance and is difficult to measure in uEVs) was not measured in this study.

ROMK weakly positively correlated with spot urinary Na^+/creatinine and spot urinary K^+/creatinine, but we detected no overall significant changes in ROMK among patients who received KCl. The visible reductions in ROMK upon KCl replacement seen in some patients were not accompanied by reduced spot urinary K^+/creatinine. Because ROMK is also expressed in the thick ascending limb of Henle's loop, it may be hard to detect K^+-dependent and aldosterone effects on ROMK, which are restricted to the aldosterone-sensitive segments. Although there is a ROMK isoform that is predominantly expressed in the distal nephron (48), the commercially unavailable antibody specific to the ROMK isoform makes it infeasible to minimize the interference with the thick ascending limb of Henle's loop expressed ROMK. Furthermore, animal studies have revealed a separate, flow-dependent BK channel that can partially compensate for inactive ROMK (49), but this was not assessed in the current study due to the limited availability of uEV material.

Estrogen in women may protect from cardiovascular and renal diseases before menopause (50, 51). Although the sex dimorphic regulation of NCC is disputed, it has been observed that estrogen restores NCC abundance in ovariectomized female rats and stimulates NCC abundance and phosphorylation (52–54). In the current study, the lack of sex-related differences in NCC and its phosphorylated forms at baseline may be related to the inclusion of women who were ≥45 years of age. By dividing women into two groups on the basis of age into "likely premenopausal" and "likely postmenopausal," we were able to demonstrate differences, possibly sex hormone related, in NCC.

In conclusion, the current study reports for the first time that acute oral KCl supplementation in patients with PA is associated with suppressed NCC abundance and phosphorylation in uEVs, despite a significant rise in plasma aldosterone, and this effect may be KCl dose dependent. These observations support (*1*) the speculation that elevation of endogenous aldosterone *per se* does not result in increased NCC abundance and phosphorylation and (*2*) that in PA, the effects of low plasma K^+ (secondary to aldosterone excess) may dominate in terms of NCC regulation.

Disclosures

R.A. Fenton reports an advisory or leadership role for the *American Journal of Physiology Renal* (associate editor) and *JASN* (editorial board member). P.A. Welling reports an advisory or leadership role for the *American Journal of Physiology* (renal editorial board) and the American Society of Physiology (chair, finance committee, and council member). All remaining authors have nothing to disclose.

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Author Contributions

D. Cowley, A. Matthews, and A. Wu were responsible for the methodology; R.A. Fenton, M. Stowasser, P.A. Welling, M.J. Wolley, and A. Wu reviewed and edited the manuscript; R.A. Fenton, P.A. Welling, and M.Stowasser were responsible for funding acquisition; M. Stowasser and M.J. Wolley were responsible for supervision; M. Stowasser, M.J. Wolley, and A. Wu were responsible for the conceptualization; M.J. Wolley and A. Wu were responsible for the investigation; A. Wu was responsible for data curation, formal analysis, project administration, resources, software, validation, and visualization, and wrote the original draft of the manuscript.

Data Sharing Statement

All data are included in the manuscript and/or supporting information.

Supplemental Material

This article contains the following supplemental material online at http://kidney360.asnjournals.org/lookup/suppl/doi:10.34067/KID.0003632022/-/DCSupplemental.

Supplemental Figure 1. Immunoblots of analyzed proteins in all participants.

Supplemental Figure 2. Changes of absolute abundances of uEV proteins among the 26 patients with PA within the 24-hour experiment period.

Supplemental Figure 3. Correlations of the analyzed proteins with biochemical parameters.

Supplemental Figure 4. Correlation between changes in plasma K^+ and plasma aldosterone in patients with PA receiving KCl replacement.

Supplemental Table 1. Detection of EV-enriched proteins in each uEV isolate, and sample inclusion in uEV analyses.

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