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Acute intravenous NaCl and volume expansion reduces NCC abundance and phosphorylation in urinary extracellular vesicles

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Key Points:
*Volume expansion induced a clear reduction in AQP2 abundance in uEVs.

*Changes in NCC and pNCC may have been primarily due to diluted post-test urine samples and a stable plasma K⁺ during the test.

Abstract:
Background: NaCl loading and volume expansion suppress the RAAS to reduce renal tubular reabsorption of NaCl and water, but effects on NCC and relevant renal transmembrane proteins that are responsible for this modulation in humans are less well investigated.

Methods: We used uEVs as an indirect readout to assess renal transmembrane proteins involved in NaCl and water homeostasis in 44 hypertensive patients with repeatedly raised aldosterone-to-renin ratios undergoing infusion of 2L of 0.9% saline over 4 hours.

Results: When measured by mass spectrometry in 13 patients, significant decreases were observed in NCC (median fold change (FC)=0.70), pendrin (FC=0.84), AQP2 (FC=0.62) and uEV markers including ALIX (FC=0.65) and TSG101 (FC=0.66). Immunoblotting reproduced the reduction in NCC (FC=0.54), AQP2 (FC=0.42), ALIX (FC=0.52) and TSG101 (FC=0.55) in the remaining 31 patients, and demonstrated a significant decrease in phosphorylated NCC (pNCC) (FC=0.49). However, after correction for ALIX, the reductions in NCC (FC=0.90) and pNCC (FC=1.00) were no longer apparent, while the significant decrease in AQP2 persisted (FC=0.62).

Conclusions: We conclude that (1) decreases in NCC and pNCC induced by acute NaCl loading and volume expansion may be due to diluted post-test urines, (2) the lack of change of NCC and pNCC when corrected for ALIX despite a fall in plasma aldosterone may be due to the lack of change in plasma K⁺ and (3) the decrease in AQP2 may be due to a decrease in vasopressin in response to volume expansion.

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Acute intravenous NaCl and volume expansion reduces sodium-chloride-cotransporter abundance and phosphorylation in urinary extracellular vesicles

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Key Points

- Volume expansion induced a clear reduction in AQP2 abundance in uEVs.
- Changes in NCC and pNCC may have been primarily due to diluted post-test urine samples and a stable plasma K\(^+\) during the test.

Abstract

Background

Sodium chloride (NaCl) loading and volume expansion suppress the RAAS to reduce renal tubular reabsorption of NaCl and water, but effects on sodium-chloride-cotransporter (NCC) and relevant renal transmembrane proteins that are responsible for this modulation in humans are less well investigated.

Methods

We used urinary extracellular vesicles (uEVs) as an indirect readout to assess renal transmembrane proteins involved in NaCl and water homeostasis in 44 hypertensive patients with repeatedly raised aldosterone-to-renin ratios undergoing infusion of 2L of 0.9% saline over 4 hours.

Results

When measured by mass spectrometry in 13 patients, significant decreases were observed in NCC (median fold change (FC)=0.70), pendrin (FC=0.84), AQP2 (FC=0.62) and uEV markers including ALIX (FC=0.65) and TSG101 (FC=0.66). Immunoblotting reproduced the reduction in NCC (FC=0.54), AQP2 (FC=0.42), ALIX (FC=0.52) and TSG101 (FC=0.55) in the remaining 31 patients, and demonstrated a significant decrease in phosphorylated NCC (pNCC) (FC=0.49). However, after correction for ALIX, the reductions in NCC (FC=0.90)
and pNCC (FC=1.00) were no longer apparent, while the significant decrease in AQP2 persisted (FC=0.62).

**Conclusion**

We conclude that (1) decreases in NCC and pNCC induced by acute NaCl loading and volume expansion may be due to diluted post-test urines, (2) the lack of change of NCC and pNCC when corrected for ALIX despite a fall in plasma aldosterone may be due to the lack of change in plasma K⁺ and (3) the decrease in AQP2 may be due to a decrease in vasopressin in response to volume expansion.
**Introduction**

Alterations in distal tubular sodium (Na$^+$) handling and extracellular fluid volume have profound effects on blood pressure, mainly due to the unique capability of the distal tubule segments to respond to hormonal stimuli and the contents of the tubular lumen.$^1$

Accumulating data have suggested that potassium (K$^+$) may be a key factor in extracellular fluid volume and blood pressure maintenance through a proposed “renal-K$^+$ switch” modulating the thiazide-sensitive sodium-chloride-cotransporter (NCC).$^2$ Reduced plasma [K$^+$] is associated with increased abundance and activity of NCC in the distal convoluted tubule,$^3$ whilst oral potassium chloride (KCl) supplementation has the capacity to limit the increase in NCC abundance induced by mineralocorticoids in both humans and mice.$^4, 5$

These alterations are also accompanied by downregulation of the Na$^+$-independent chloride (Cl$^-$)/bicarbonate (HCO$_3^-$) exchanger pendrin.$^6$

In contrast to the negative regulatory role of K$^+$ on NCC, we and others have suggested that aldosterone plays a stimulatory role.$^5, 7, 8$ In patients with primary aldosteronism (PA) undergoing 4-day administration of exogenous mineralocorticoid was associated with increases in NCC and phosphorylated NCC (pNCC), whereas plasma K$^+$ inversely correlated with NCC and pNCC at baseline.$^5$ In another group of patients, although increases in NCC and pNCC were not observed, the inverse correlations with plasma K$^+$ were replicated.$^6$

However, interpretation of the findings of the two studies was complicated by the fact that they involved (1) exogenous mineralocorticoid administration, (2) KCl supplementation and (3) oral NaCl loading.

PA is a common specifically treatable and potentially curable form of hypertension, characterised by excessive and autonomous production of aldosterone by the adrenal glands. Seated saline suppression testing (SSST) is a highly sensitive, reliable yet relatively simple
method of confirmatory testing that has a low rate of inconclusive results when compared with the more time consuming and laborious fludrocortisone suppression test.\textsuperscript{9, 10} During SSST, hypertensive subjects with elevated aldosterone-to-renin ratios (ARRs) undergo intravenous infusion of 2-litre of 0.9% saline over 4 hours while patients are maintained in the seated position. In subjects without PA, the acute NaCl loading and volume expansion during SSST, by suppressing renin (and consequently the chronic aldosterone regulator angiotensin II), leads to suppression of plasma aldosterone, whereas in patients with PA, plasma aldosterone remains unsuppressed.

uEVs are a tool to assess renal epithelial cell function in humans.\textsuperscript{7, 11} A recent large-scale unbiased analysis identified uEV proteins that track the abundance of the parent protein in the kidney,\textsuperscript{12} further supporting the reliability of using uEV protein changes to monitor specific physiological responses and disease mechanisms.

In the current observational study, we took advantage of the more simplified nature of the SSST, which involved only NaCl loading and used the non-invasive approach of examining uEVs,\textsuperscript{13-15} to (1) explore the effects of NaCl loading and volume expansion on renal transmembrane proteins involved in salt and volume homeostasis using quantitative mass spectrometry (LC-MS/MS); (2) validate LC-MS/MS findings in a larger sample size by immunoblotting; and (3) define the role (if any) of NaCl loading alone on NCC abundance and phosphorylation in hypertensive patients with raised ARRs.

**Methods**

Detailed methods were demonstrated in Supplementary Methods. Briefly, from April 2017 to August 2020, a total of 44 (F29/M15) hypertensive patients with raised ARRs were invited and all agreed to participate. All participants underwent SSST to confirm or exclude the diagnosis of PA. At least four weeks prior to SSST, medications affecting plasma
aldosterone and renin levels were withdrawn and replaced by other anti-hypertensive medications including verapamil, prazosin or doxazosin, moxonidine and/or hydralazine. Patients undergoing SSST were admitted to hospital to ensure the dietary (normal hospital diet) and posture requirement were met and to facilitate monitoring of plasma K⁺ and other parameters. SSST involved intravenous infusion of 2-litre of 9% saline over 4 hours in a seated position, and assessment of plasma aldosterone concentration, direct renin concentration and cortisol concentration at baseline and at the end of the infusion.¹⁰, ¹⁶, ¹⁷ Mid-stream urines were collected before SSST at 7AM (Basal) and 1 hour post completion (Post) of the saline infusion. uEVs were isolated using progressive ultracentrifugation as previously described.⁵ uEVs were characterised, and analysed by quantitative tandem mass tag (TMT) labelled LC-MS/MS,⁶ ¹⁸ or immunoblotting.

-Ethical issues

The SSST was performed in the Hypertension Units of Princess Alexandra Hospital, Brisbane, Australia. The laboratory investigations were performed in the Endocrine Hypertension Research Centre, The University of Queensland Diamantina Institute, Brisbane, Australia and the Department of Biomedicine, Aarhus University, Denmark. Ethical approval was granted by the Metro South Human Research Ethics Committee (Clinical Trials Repository ID: CT-2018-CNT-03504-1 v1).

-Bioinformatic and statistical analyses

Calculations were processed with R. Overlap analyses were performed using Vesiclepedia and ExoCarta protein databases to compare the mass spectrometry dataset with other human urine studies and to identify EV-enriched proteins. A rat renal transporter protein database was used to identify renal transmembrane proteins.¹⁹ Gene ontology analysis was performed by ClueGO plugin (v2.5.5) in the Cytoscape environment (v3.7.2), and gene lists
corresponding to 878 DEPs were used as input. Gene Ontology (GO) terms were updated on 11th June 2020.

In TMT-labelled LC-MS/MS, protein ratios obtained with the aid of the universal control channel TMT 126 were log2 transformed. In immunoblotting, protein absolute abundances were analysed by Image J software. To minimise operation errors occurring during gel loading and transferring, relative protein abundance was applied to allow comparison between blots, which was determined as dividing the protein absolute abundance by a ratio that was obtained from normalisation of EV marker protein Apoptosis-Linked Gene 2-Interacting Protein X (ALIX) in the control sample loaded on each blot. The relative protein abundance was then log10 transformed.

For paired comparisons before and after SSST, Wilcoxon tests were performed to compare the differences of biochemical parameters, and T-tests were performed to compare the differences of protein ratios/relative abundance. In TMT-labelled LC-MS/MS, a differentially expressed protein (DEP) was identified as that with \( p < 0.05 \), false discovery rate (FDR) < 0.1 and fold change of \( \geq 1.20 \) or \( \leq 0.83 \). Pearson’s correlations were assessed to seek correlations between protein ratios/abundances and biochemical parameters. A \( p \) value < 0.05 was considered statistically significant. Protein data are presented as median [range], unless stated otherwise.

**Results**

-Participants’ clinical features during SSST

A total of 44 participants (F29/M15) were recruited (screening features and use of anti-hypertensive drugs are listed in Supplementary Table S1) and completed SSST. Numbers of participants at each stage of analyses is summarised in Supplementary Figure S1. SSST was
positive in 34 (F19/M15) thereby confirming PA, and was negative in 10 (F10/M0) in which PA was excluded and hereafter designated low renin essential hypertension (LRH).

Participants’ clinical characteristics and biochemical changes during SSST is summarised in Table 1. Significant decreases in plasma concentrations of aldosterone and renin were observed in both PA and LRH subjects demonstrating the suppressive effect of SSST on the RAAS. Plasma ARR decreased but its decrease in PA was not significant, reflecting the autonomous aldosterone overproduction in PA. A reduction in plasma cortisol in both PA and LRH occurred between 7AM and the completion time of SSST (12PM) in keeping with the known fall in adrenocorticotropic hormone as part of its normal circadian rhythm during this time period. There were no changes in plasma \([K^+]\) in patients either with PA or LRH, but a significant increase in plasma \([Cl^-]\) (probably due to infusion of NaCl) and a non-significant trend toward a decrease in plasma \([HCO_3^-]\) in both PA and LRH subjects. Plasma copeptin fell during SSST (significantly among the total cohort and the PA subgroup), reflecting a decrease in circulating arginine vasopressin induced by volume expansion during SSST. Spot urine creatinine fell significantly in both PA and LRH subjects. Blood pressures during SSST were measured in all but one (Pt#39). Systolic and diastolic blood pressures did not change significantly in the 34 (F19/M15) with PA, the 10 (F10/M0) with LRH or in the combined cohort of 44.

- **Characterisation of uEVs**

Due to the limited amount of uEVs obtained from participants, nine uEVs isolated from two healthy volunteers at different times on multiple days were characterised by both nanoparticle tracking analysis (NTA) and the presence of marker proteins using immunoblotting. The diameter of the uEV particles from the nine uEVs ranged overall from 25.5 to 999.5 nm, with mean particle size for each of the nine samples ranging from 218.9±1.6 to 341.7±5.9 and mode particle size from 139.9±7.0 to 194.9±6.7 nm (Figure 1A-C and Supplementary Table...
S2). Immunoblotting detected the most frequently used uEV marker ALIX in all the nine uEV samples, and tumour susceptibility gene 101 (TSG101) and tetraspanin CD9 (CD9) in most samples (Figure 1D).

Patients’ uEV samples were characterised by the presence of EV-enriched proteins. LC-MS/MS quantified 99 proteins isolated from 13 (PA10/LRH 3, F8/M5) patients’ uEVs that were among the list of the top 100 EV-enriched proteins published on Vesiclepedia, including the widely used EV markers (Figure 1E and Supplementary Spreadsheet). Immunoblotting detected ALIX in uEVs isolated from 31 (F21/M10, PA24/LRH7) subjects, and detected TSG101 from 27 (F17/M10, PA22/LRH5) subjects (Figure 3 and Supplementary Figure S5). We observed a shift in the ALIX bands in multiple samples in immunoblots whilst no size shifts for other proteins in the same samples. Despite ALIX truncation by the ESCRT machinery, our main hypothesis is that this may be tamm-horsfall protein-related, which was also observed in a recent study.

P -Proteomic analyses

Among the 13 participants subjected to TMT-labelled LC-MS/MS, a total of 3,307 proteins were identified, of which 3,007 proteins were quantifiable (Supplementary Spreadsheet, a simplified version can be directly accessed at http://interpretdb.au.dk/database/SSST/SSST_proteome.html). Comparison of proteins quantified in the current study and other human uEV and urinary exosome databases demonstrated 79.0% overlap with the uEV database Vesiclepedia, 72.7% overlap with urinary exosome database ExoCarta (Figure 2A), and 104 renal transporter proteins were identified in the uEVs (Figure 2A).

A total of 878 DEPs were identified (Figure 2B and Supplementary Spreadsheet), with 636 increased and 242 decreased in abundance after SSST. Of these, 294 DEPs were quantified in
all paired uEV samples, but there were no apparent differences between PA and LRH subjects as demonstrated using an unsupervised hierarchical clustering heat map (Supplementary Figure S2). Of the 294 DEPs, 29 were identified as EV-enriched proteins (Figure 1E) and 12 were renal transmembrane proteins (Table 2). Decreases in NCC, pendrin and Aquaporin-2 were notable due to their involvement in NaCl and water homeostasis. However, significant decreases in the widely used uEV markers (e.g., ALIX, fold change (FC)=0.65 [0.43, 1.60]; TSG101, FC=0.66 [0.39, 1.40], CD63, FC=0.77 [0.34, 1.29]) were also observed, implying changes in exosomal biogenesis and secretion. Although ENaC subunits were not detected in this experiment, prostasin was identified in all uEV samples, which was an assumed indicator of full ENaC activity/cleavage,22,23 but its level did not change during SSST.

GO enrichment suggested upregulated DEPs were closely associated with cell components including the mitochondrial matrix (22.03%), whist the downregulated DEPs associated with cytoplasmic vesicles (38.46%). In the biological process category, the upregulated DEPs were associated with establishment of location in cell (25.32%), whilst downregulated DEPs were associated with secretion by cell (31.67%) (Supplementary Figure S3).

Among the 3 transmembrane DEPs of interest, plasma [K+] negatively correlated with pendrin ($R^2=0.23$, $p=0.01$), plasma copeptin positively correlated with aquaporin 2 (AQP2) ($R^2=0.7$, $p<0.01$), but of caution these correlations were based on only eight plasma copeptin measurements in five participants We did not observe clear correlations between NCC and plasma aldosterone or K+ unlike in our previous study using immunoblotting (Supplementary Figure S4).5

-Immunoblotting validation of decreases in NCC and AQP2
Due to limited amount of uEVs, immunoblotting measured abundances of NCC, pNCC and ALIX in uEVs isolated from 31 subjects (F21/M10, PA24/LRH7), and abundances of AQP2 and TSG101 in uEVs from 19 (F12/M7, PA17/LRH2) and 27 (F17/M10, PA22/LRH5) subjects respectively (Figure 3 and Supplementary Figure S5). Immunoblotting reproduced the significant decreases in the relative abundances of NCC (FC= 0.54 [0.02, 3.72], \( p<0.001 \)) and AQP2 (FC=0.42 [0.009, 2.00], \( p=0.003 \)) observed with LC-MS/MS. A reduction was also observed in the abundance of pNCC (FC=0.49 [0.02, 2.15], \( p<0.001 \)), whilst no apparent change was detected in the pNCC-to-NCC ratio (FC= 1.10 [0.11, 4.20], \( p=0.31 \)) (Figure 4). The accompanying decreases in the uEV marker ALIX (FC= 0.52 [0.05, 7.29], \( p<0.001 \)) and TSG101 (FC= 0.55 [0.13, 3.63], \( p<0.0001 \)) were also reproduced (Figure 4), which again raised the possibility that decreases in the abundances of proteins of interest may be due to decreased uEV concentration.

After correction for spot urine creatinine concentrations, decreases were replaced with small increases for NCC (FC=1.72 [0.03, 56.27], \( p=0.06 \)), pNCC (FC=1.59 [0.04, 20.18], \( p=0.06 \)), ALIX (FC=2.00 [0.08, 11.86], \( p<0.01 \)) and TSG101 (FC=1.62 [0.21, 8.96], \( p=0.01 \)), and AQP2 (FC=0.99 [0.07, 5.18], \( p=0.94 \)) did not change (Figure 4). After correcting relative protein abundances for the uEV marker ALIX, the decreases in NCC (FC=0.90 [0.13, 7.19], \( p=0.31 \)) and pNCC (FC=1.00 [0.16, 5.04], \( p=0.52 \)) were abolished, whilst the decrease in AQP2 remained significant (FC= 0.62 [0.03, 3.79], \( p=0.04 \)) (Figure 4).

**Plasma \([K^+]\) inversely correlated with NCC and AQP2**

Plasma \([K^+]\) inversely correlated with NCC abundance (\( R^2=0.07, p=0.02 \)) (Figure 5), and this correlation remained after correction for the uEV marker ALIX (\( R^2=0.08, p=0.02 \)) but not after correction for spot urine creatinine concentration (\( R^2=0.06, p=0.07 \)). Besides, plasma \([K^+]\) inversely correlated with AQP2 (\( R^2=0.22, p=0.002 \)), and this correlation remained
significant after correction for ALIX ($R^2=0.26, p<0.001$) and correction for urine creatinine ($R^2=0.21, p=0.007$) (Figure 5). No clear correlation was detected between plasma $[K^+]$ and pNCC abundance unlike in our previous observations (Supplementary Figure S6).

Plasma aldosterone appeared to positively correlate with AQP2 ($R^2=0.11, p=0.04$) and the ALIX corrected abundance of AQP2 ($R^2=0.10, p=0.04$). Plasma copeptin positively correlated with AQP2 ($R^2=0.34, p<0.001$) and the ALIX and urine creatinine corrected AQP2 ($R^2=0.36, p<0.001$ and $R^2=0.21, p<0.01$, respectively) (Figure 5). Plasma $[Cl^-]$ showed trends towards positive correlations with creatinine corrected pNCC ($R^2=0.06, p=0.08$), and ALIX corrected NCC ($R^2=0.05, p=0.09$) and pNCC ($R^2=0.06, p=0.06$) (Supplementary Figure S6).

**Discussion**

The primary aim of the current study was to use large-scale proteomic techniques to investigate the effect of acute NaCl loading and volume expansion on renal transmembrane proteins in uEVs from hypertensive patients with raised ARRs. Although we did not have sufficient quantity of all uEV samples to perform transmission electron microscopy, the size distribution assessment by NTA, coupled to the identification of widely used uEV markers including ALIX and TSG101 suggested successful uEV isolation using the progressive ultracentrifugation. This was also supported by the non-biased LC-MS/MS data where of the over 3000 proteins identified, 99 were identified as EV-enriched proteins and over 70% of them had previously been found to be present in uEVs and exosomes.

Of the proteins determined by quantitative LC-MS/MS to be altered in abundance during SSST, reductions in NCC, pendrin and AQP2 were of interest. However, several uEV markers were also differentially expressed during SSST, suggesting that the reductions in these proteins may be due to alterations in actual uEV biogenesis or excretion rates. To address this possibility, we validated the effects of SSST on AQP2, NCC (and its
phosphorylated form), and the uEV markers ALIX and TSG101 using immunoblotting. We did not validate pendrin due to lack of antibodies cross-reacting with human pendrin. In line with the LC-MS/MS data, in both PA and LRH subjects there were decreases in NCC, AQP2, ALIX and TSG101, and pNCC additionally showed a reduction at completion of the SSST, although the findings in LRH subjects need validation due to small numbers.

In the Hypertension Units of Princess Alexandra Hospital, during SSST, anti-hypertensive drugs that have the potential to significantly affect the measurement of plasma ARR were withdrawn, at least four weeks before SSST for diuretics (including spironolactone), and at least two weeks before SSST for β-blockers, clonidine, methyldopa, NSAIDs, ACE inhibitors, ARBs, and dihydropyridine calcium blockers. Other anti-hypertensive medications that have lesser effects on the ARR, including verapamil, prazosin or doxazosin, moxonidine and/or hydralazine, were commenced where necessary to ensure ongoing control of hypertension. Therefore, the likelihood that anti-hypertensive medications may have had significant effects on the sodium channels is small.

There was a large reduction observed in urine creatinine concentration. We therefore corrected relative protein abundances for urine creatinine concentrations, and found that the apparent decreases in NCC, pNCC, ALIX and TSG101 were replaced with mild increases and the change in AQP was no longer evident. However, correction for creatinine concentration in spot urines does not address the influence of variable uEV recovery/sedimentation rates during progressive ultracentrifugation. We then performed correction for the uEV marker protein ALIX. This resulted in abolition of the decreases in NCC and pNCC, whilst the decrease in AQP2 remained, and the changes in the three channels were reproduced when predicting their total contents in the collected urine by multiplying each relative abundance to total urine volume (Supplemental Figure S7). These
observations raise the possibility that decreases in NCC and pNCC in uEVs observed in LC-MS/MS and immunoblots are due to reductions in uEVs, either because the concentration of uEVs is reduced after SSST (supported by the reduced urine creatinine concentration), or less uEVs are excreted during SSST (supported by reduced abundance of EV markers). A recent study demonstrated that water loading reduced the abundance per unit volume of EV markers but increased the amount of tamm-horsfall protein recovered in uEVs. Acute saline loading-induced increased urine volume may also result in greater excretion of albumin in the post sample compared to the baseline condition. Given that the total protein from each sample loaded on MS (6.4 µg) or WB (20 µg) was the same, it is therefore not surprising that the readout of uEV proteins was lower after SSST. Findings of a recent study comparing uEV quantification methods suggested that urine creatinine can replace the need for uEV quantification to normalise spot urines. In the current study, the abundance of widely used EV markers, whether quantified by MS or immunoblotting, positively correlated with spot urine creatinine concentration (Supplementary Figure S8).

The apparent lack of changes in ALIX-corrected NCC and pNCC despite the fall in endogenous aldosterone may be due to the lack of change in plasma K⁺ during SSST. Although aldosterone and its analogues were originally thought to be major regulators of NCC, plasma [K⁺] is suggested a more potent regulator of NCC abundance and phosphorylation. Sufficient dietary K⁺ supplementation to maintain normokalemia in mice during aldosterone infusion reduced plasma membrane NCC. In the current study, the weak inverse correlation of plasma [K⁺] with NCC is consistent with animal studies. The low R² value of the correlation of plasma K⁺ with ALIX-corrected NCC and the unclear association between plasma [K⁺] and ALIX-corrected pNCC in the current study may reflect the fact that, unlike in our previous studies, almost all patients’ plasma [K⁺] fell within the
normal range (3.5-5.2 mmol/L) with considerably less variation among samples, resulting in a lower power to detect their relationship.

A fall in ALIX-corrected AQP2 may reflect a fall in plasma vasopressin induced by water loading,\(^{36}\) as evidenced by the significant decrease in plasma copeptin in both PA and LRH subjects. Reduction in AQP2 abundance may also have been due to reduced aldosterone, which is a potent stimulator of AQP2 expression independently of ANGII.\(^{37}\) Surprisingly, there was a negative correlation between plasma [K\(^+\)] and creatinine corrected AQP2 (determined by immunoblotting). Plasma [K\(^+\)] usually positively correlates with kidney levels of AQP2, with K\(^+\) deficiency rapidly resulting in nephrogenic diabetes insipidus (NDI) due to autophagic degradation of AQP2.\(^{38-40}\) It is possible that the increased uEV levels of AQP2 when plasma [K\(^+\)] are lower represents a cellular mechanism to remove AQP2 from principal cells during hypokalemia and may be linked to the process of autophagy.\(^{41,42}\)

When measured by LC-MS/MS, the negative correlation between plasma [K\(^+\)] and pendrin is consistent with our previous report.\(^{5,6}\) There are additional associations of the remaining 9 renal transmembrane DEPs with biochemical factors (Supplementary Figure S4). The increases in VDACs and their positive correlations with plasma [Cl\(^-\)] may reflect the transport of Cl\(^-\) across the mitochondrial membranes and plasma membrane due to infusion of NaCl. Besides, the correlations, detected by LC-MS/MS and immunoblotting, between plasma [HCO\(_3\)] and several of the proteins examined require validation. The fact that [HCO\(_3\)] were much lower than the normal range in some patients despite being clinically well raises the possibility of a technical issue, as processing of plasma samples for [HCO\(_3\)] measurement was delayed and some samples were subjected to freeze-defrost cycles before measurement.

Admittedly, the diverse origins and dynamic molecular composition of uEVs present an enormous analytical challenge. Therefore, it remains uncertain as to what extent the uEV
isolation and measurement approaches used in the current study and the data that they yield are able to truly reflect disturbances to physiological processes that may occur across a range of disease scenarios. Although uEV analysis has been demonstrated to be a reliable tool to monitor specific physiological responses, methods of uEV quantification and normalization need further optimisation and standardization to foster scientific advances in uEV research and successful translation into clinical practice.

In summary, this is the first study to quantify changes in the protein profile of uEVs in response to acute NaCl loading and volume expansion-induced RAAS inhibition in hypertensive subjects with repeatedly elevated ARRs. Volume expansion induced a clear reduction in AQP2 abundance, but changes in NCC and pNCC may have been primarily due to diluted post-SSST urine samples and a stable plasma [K⁺] during the test despite a fall in aldosterone levels. A study in hypertensive cohort with raised ARRs whose plasma [K⁺] decreased post SSST, is required to further elucidate if plasma [K⁺] contributes to the variations in NCC abundance and phosphorylation.

Disclosures

R. Fenton reports the following: Advisory or Leadership Role: Associate editor for American Journal of Physiology Renal, Editorial board member of J Am Soc Neph, Editorial board member of (2008-), Editorial board member of PLOS One (2011-), Editorial board member of Nature Scientific Reports (2016-). P. Welling reports the following: Research Funding: NIH, LeDucq Foundation; Honoraria: American Physiological Society; and Advisory or Leadership Role: American Journal of Physiology, Renal Editorial Board; Chair, Finance Committee, American Physiological Society; Chair, Kidney Molecular Biology and Development, NIH. The remaining authors have nothing to disclose.
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Author contributions

Aihua Wu: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Software; Validation; Visualization; Writing - original draft. Martin Wolley: Conceptualization; Funding acquisition; Investigation; Supervision; Validation; Writing - review and editing. Qi Wu: Data curation; Formal analysis; Investigation; Methodology; Resources; Software; Writing - review and editing. Diane Cowley: Data curation; Methodology. Johan Palmfeldt: Data curation; Methodology; Resources; Software. Paul Welling: Funding acquisition; Validation; Writing - review and editing. Robert Fenton: Funding acquisition; Investigation; Methodology; Software; Supervision; Validation; Writing - review and editing. Michael Stowasser: Conceptualization; Funding acquisition; Investigation; Supervision; Validation; Writing - review and editing.

Data sharing statement

The LC-MS/MS proteomics data (Supplementary Spreadsheet) have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier
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Table 1. Participants’ characteristics and changes in biochemical factors during SSST.

| Characteristic     | Normal range in adults | Value          |
|--------------------|------------------------|----------------|
| **Screening measurements (n=44)** |                        |                |
| Age (year)         |                        | 51.9±11.0      |
| Female/Male (n/n)  |                        | 29/15          |
| Weight (kg)        |                        | 89.3±21.2      |
| Height (cm)        |                        | 167.0±9.6      |
| BMI (kg/m^2)       | 18.5-24.9              | 31.9±6.4       |
| Number of anti-HTN drugs |                    | 1.7±1.1        |
| SBP/DBP (mmHg)     | <140/90                | 146.3±18.0     / 86.6±14.9 |
| eGFR (ml/min)      | >60                    | 96.7±12.7      |
| Plasma Creatinine (μmol/L) |                | 45-90          / 67.2±15.8 |
| Diagnosis          |                        | 34/10          |

| Measurements during SSST | PA (n=34, F19/M15) | LRH (n=10, F10/M0) |
|--------------------------|--------------------|--------------------|
|                         | Basal | Post | p-value | Basal | Post | p-value |
| Plasma aldosterone (pmol/L) | 100-950 | 615.9±515.0 | 426.9±421.2 | 9.1E-04 | 479.0±327.0 | 129.9±85.5 | 0.002 |
| Plasma renin (mU/L)      | 8-40  | 3.5±2.1 | 2.8±1.8 | 0.002 | 7.3±7.4 | 4.2±3.8 | 0.01 |
| Plasma ARR (pmol/mU)     | 2-75  | 231.0±305.3 | 188.2±150.6 | 0.40 | 128.1±128.4 | 51.9±48.3 | <0.01 |
| Plasma cortisol (nmol/L) | 8AM 140-640 4PM 80-440 | 325.1±99.1 | 164.0±57.5 | 2.8E-06 | 358.8±179.9 | 149.4±65.7 | 0.002 |
| Plasma K⁺ (mmol/L)       | 3.5-5.2 | 3.67±0.42 | 3.74±0.37 | 0.20 | 3.93±0.36 | 3.90±0.22 | 0.73 |
| Plasma Cl⁻ (mmol/L) *    | 95-110 | 96.3±2.8 | 100.0±3.2 | 2.1E-06 | 96.0±2.5 | 100.6±3.5 | 0.004 |
| Plasma HCO₃⁻ (mmol/L) *  | 22-32  | 23.3±6.0 | 22.4±6.0 | 0.10 | 22.7±4.9 | 21.3±5.3 | 0.08 |
| Plasma Copeptin (pmol/L) ** | 8.8±6.8 | 6.6±6.5 | 0.02 | 5.2±4.6 | 3.1±1.8 | 0.25 |
| SBP (mmHg)               | <140  | 142.0±24.6 | 146.6±20.7 | 0.17 | 139.3±20.4 | 141.0±15.1 | 0.65 |
| DBP (mmHg)               | <90   | 83.9±12.9 | 85.8±13.3 | 0.29 | 82.1±13.8 | 78.8±11.7 | 0.55 |
| Spot urine creatinine (mg/dL) *** | 108.7±45.6 | 35.3±22.9 | 9.3E-10 | 94.8±65.2 | 36.2±21.1 | 0.02 |

**Abbreviations:** SSST, seated saline suppression testing; n, sample size; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; eGFR, estimated Glomerular filtration rate; LRH, low renin hypertension; PA, primary aldosteronism; F, female; M, male. Basal, baseline measurement prior to SSST commencement; Post, measurement at SSST completion; p-value, paired Wilcoxon test p value (Post/Basal); * paired plasma Cl⁻ and HCO₃⁻ were measured in 41 participants (PA31, F18/M13; LRH10, F10/M0); ** paired plasma copeptin were measured in 33 participants (PA25, F14/M11; LRH8, F8/M0); paired spot urine creatinine was measured in 39 participants (PA32, F18/M14; LRH7, F7/M0). Values are the mean±SD unless otherwise noted.
Table 2. Differentially expressed renal membrane proteins quantified in all samples during SSST (n=13, F8/M5, PA10/LRH3).

| No. | Accession | Protein Description | Alternative Name | Gene Symbol | Fold change (Post/Basal) median[range] | Trend | p-value | FDR |
|-----|-----------|---------------------|------------------|-------------|----------------------------------------|-------|---------|-----|
| 1   | P55017-2  | Isoform 2 of Solute carrier family 12 member 3 | NCC | SLC12A3 | 0.70 [0.50, 1.82] | Decrease | 0.026 | 0.091 |
| 2   | P21796    | Voltage-dependent anion-selective channel protein 1 | VDAC-1 | VDAC1 | 1.49 [0.58, 4.24] | Increase | 0.030 | 0.098 |
| 3   | O95833    | Chloride intracellular channel protein 3 | CLIC3 | CLIC3 | 1.27 [0.85, 2.08] | Increase | 0.003 | 0.023 |
| 4   | P45880-1  | Isoform 1 of Voltage-dependent anion-selective channel protein 2 | VDAC-2 | VDAC2 | 1.47 [0.82, 3.22] | Increase | 0.003 | 0.022 |
| 5   | Q9Y277    | Voltage-dependent anion-selective channel protein 3 | VDAC-3 | VDAC3 | 1.58 [0.65, 4.24] | Increase | 0.003 | 0.022 |
| 6   | O43511    | Pendrin | Pendrin | SLC26A4 | 0.84 [0.55, 1.18] | Decrease | 0.007 | 0.040 |
| 7   | P05141    | ADP/ATP translocase 2 | ANT2 | SLC25A5 | 1.88 [0.74, 4.39] | Increase | 0.005 | 0.033 |
| 8   | Q9C0H2-4  | Isoform 4 of Protein tweety homolog 3 | TTYH3 | TTYH3 | 0.61 [0.35, 1.14] | Decrease | 0.000 | 0.008 |
| 9   | P41181    | Aquaporin-2 | AQP2 | AQP2 | 0.62 [0.24, 1.39] | Decrease | 0.004 | 0.026 |
| 10  | Q9NQA5    | Transient receptor potential cation channel subfamily V member 5 | ECaC1 | TRPV5 | 0.77 [0.40, 1.11] | Decrease | 0.002 | 0.017 |
| 11  | Q9NRX2    | H+ /sialic acid cotransporter Sialin | Sialin | SLC17A5 | 0.63 [0.25, 1.61] | Decrease | 0.010 | 0.049 |
| 12  | Q00325    | Phosphate carrier protein, mitochondrial | PTP | SLC25A3 | 1.66 [0.79, 4.56] | Increase | 0.003 | 0.025 |

**Abbreviations:** SSST, seated saline suppression testing; n, sample size; F, female; M, male; PA, primary aldosteronism; LRH, low renin hypertension; No., number; p-value, paired t-test (Post/Basal); FDR, false discovery rate.
**Figures and Figure Legends**

**Figure 1:** Characterisation of uEVs. A, Screenshot from a diluted uEV sample (1/1000) revealing a range of particle sizes; B, Concentrations (particle/mL) of the uEV sample in screenshot A (expressed as averaged finite track length adjustment concentration, the remaining eight uEV samples displayed in box); C, NTA depicting the nanoparticle size density of the uEV sample in screenshot A; D, Immunoblotting of uEVs-enriched proteins (ALIX, TSG101 and CD9) in nine different uEVs samples isolated from two healthy volunteers collected at different time on multiple days; E, Volcano plot of uEV-enriched proteins detected by LC-MS/MS.

**Figure 2.** Proteomic analyses of uEV proteins. A. Venn diagram summarising quantified proteins (represented as A) and their overlaps with Vesiclepedia (represented as B), Exocarta (represented as C) and a renal transport protein database (represented as D); B, Volcano plot depicting differentially abundant proteins affected by SSST in 13 participants. Proteins quantified in ≥4 samples were used as input. The -log10 p value (paired t-test with 95% CI) is plotted against the mean log2 fold change (Mean of [log2 Post ratio - log2 Basal ratio]). The non-axial horizontal dotted line denotes p=0.05, which is the significant threshold (prior to logarithmic transformation). The two grey non-axial vertical dotted lines denote fc (fold change) of 1.20 or 0.83 during SSST. The black dots represent proteins with p<0.05, FDR<0.1 and fc of ≥1.20 or ≤0.83 during SSST to be the differentially expressed proteins (DEPs), and the grey dots represent proteins not defined as DEPs.

**Figure 3.** Line plots summarising WB detected proteins in absolute abundances (Absolute.Ab) of NCC, pNCC, AQP2, ALIX and TSG101 in uEVs from all subjects, as a summarised presentation of Supplemental Figure S5.
**Figure 4.** Boxplots of statistical differences in relative abundance of analysed proteins without and with correction for urine creatinine or ALIX, and change in pNCC/NCC ratio during SSST. PA, primary aldosteronism; LRH, low renin essential hypertension; Dx, SSST diagnosis; Rel.Ab, protein relative abundance; Rel.Ab/Cr, protein relative abundance corrected for spot urine creatinine concentration; Rel.Ab/ALIX, protein relative abundance corrected for ALIX; Cr, urine creatinine concentration.

**Figure 5.** Notable correlations between plasma variables and immunoblotting analysed proteins of interest in all participants. The first two columns demonstrate negative correlations between plasma K$^+$ and NCC, ALIX-corrected NCC (NCC/ALIX), AQP2, ALIX-corrected AQP2 (AQP2/ALIX) and creatinine corrected AQP2 (AQP2/Cr); The third column shows positive correlations between plasma aldosterone (Aldo) and AQP2 and ALIX-corrected AQP2 (AQP2/ALIX); the last column shows positive correlations between plasma copeptin and AQP2, ALIX-corrected AQP2 (AQP/ALIX) and creatinine corrected AQP2 (AQP/Cr).
Figure 1
Figure 5

- Plasma K+ vs. NCC and AQP2
- Plasma Aldo vs. AQP2
- Plasma Copeptin vs. AQP2
- NCC/ALIX vs. K+
- AQP2/ALIX vs. K+
- AQP2/ALIX vs. Aldo
- AQP2/ALIX vs. Copeptin
- AQP2/Cr vs. K+
- AQP2/Cr vs. Copeptin

Statistical values:
- R² and p-values provided for each graph.
Supplementary Materials

Wu and Wolley et al., Acute intravenous NaCl and volume expansion reduces sodium-chloride-cotransporter abundance and phosphorylation in urinary extracellular vesicles

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Supplementary Methods

• Recruitment

Hypertensive patients who were admitted for SSST in the Hypertension Unit of Princess Alexandra Hospital were invited to participate and provide informed written consent. A total of 44 (F29/M15) patients were invited and all agreed to participate. All subjects were hypertensive with repeatedly raised ARRs and underwent SSST to confirm or exclude the diagnosis of PA.

• Sample size

We previously reported several uEV proteins increased over 2.5-fold in responding to alterations of mineralocorticoid and salt loading, and plasma aldosterone to be suppressed on at least of 101 pmol/L in 24 patients with PA subjects. For proteins that are detectable by MS and WB, we accepted a $p<0.05$ with 80% power using two-tailed test for bidirectional result. Due to the co-isolation and fragmentation of the isobaric precursor ions, a TMT multiplex labelling strategy reduces missing data points, providing high-quality data for statistical analysis from a limited number of clinical samples (generally one peptide with $\leq 5$ replicates per condition) [20, 21]. Assuming SSST would induce at least a 50%-fold change, we required at least 10 subjects analysed on MS and 16 subjects analysed on WB.

• The SSST

At least four weeks prior to SSST, medications affecting plasma aldosterone and renin levels were withdrawn and replaced by other anti-hypertensive medications (e.g., verapamil, prazosin or doxazosin, moxonidine and/or hydralazine). Patients undergoing
SSST 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. An infusion of 2 L of 0.9% saline over 4 hours was commenced at 8AM, 30 min after assuming a seated position. Two aliquots of bloods were collected just before 8AM (baseline) and at completion of the infusion at 12 noon (post-SSST). Clinical routine measurements of plasma aldosterone (by LC-MS/MS), direct renin concentration (by chemiluminescent immunoassay), cortisol (by immunoassay) and K⁺ levels were performed by Pathology Queensland Laboratory right after the SSST. Due to limited amounts of bloods, plasma concentrations of Cl- and HCO3- were analysed by Pathology Queensland Laboratory in 41 participants, including 31 (F18/M13) PA and 10 (F10/M0) LRH subjects, plasma copeptin was measured in 33 participants (PA25, F14/M11; LRH8, F8/M0). Blood pressure was recorded during SSST. Demonstration of aldosterone production that is relatively autonomous of its chronic regulator angiotensin II requires failure of plasma aldosterone to suppress to below 162 pmol/L, provided that direct renin concentration at completion was less than 8.4 mU/L and plasma cortisol concentration was lower at completion than that basally.

- Urine collection and isolation of uEVs

Participants were provided with two sterilised 200ml containers for urine collection. 50-200 ml of mid-stream second morning urine were collected before SSST at 7AM (basal) and 1 hour post completion (post) of the saline infusion. Collected urine samples were immediately treated with protease inhibitor cocktail (Roche cOmplete, EDTA-free) and phosphatase inhibitors (Sigma-Aldrich, Pierce™) before aliquoting and freezing at -80°C. Nine participants provided an additional urine collection at 8AM on the day before SSST
to be used as a baseline sample, as participants felt they would be unable to produce enough urine volume on the day of SSST. Due to limited spot urine samples, spot urine creatinine concentration was measured in 39 participants (PA32, F18/M14; LRH7, F7/M0) using a creatinine urinary detection assay kit (EIACUN, Invitrogen). uEVs were isolated using progressive ultracentrifugation techniques with 200 mg/mL dithiothreitol treatment. For LC-MS/MS the obtained uEVs were resuspended in 200 μl uEV isolation buffer containing 1x phosphate-buffered saline (PBS), protease and phosphatase inhibitors and 0.5% SDS. For immunoblotting analysis, uEVs were resuspended in approximately 110 μl of 1x PBS containing 0.5% SDS. Resuspended uEVs were ultrasonic-homogenised for 5 cycles of 10 seconds on/off on ice before being frozen at -80°C pending subsequent analyses.

- Characterisation of uEVs

uEVs were characterized by size distribution measured by nanoparticle-tracking analysis (NTA) and the presence of marker proteins of EV (e.g., ALIX, TSG101). Due to the limited amount of uEVs obtained from participants, nine uEVs isolated from two healthy volunteers at different times on multiple days were characterised by both NTA and the presence of marker proteins using immunoblotting, and patients’ uEVs were characterised by the presence of marker proteins by LC-MS/MS and immunoblotting. For NTA, a NanoSight NS500 instrument (Nanosight Ltd, Amesbury, UK) with NanoSight NTA v3 software was used. Before each session, the acquisition parameter settings were determined using the NTA latex standard (Malvern Polystyrene Latex Microsphere 100nm) in a 1/250 dilution in ultrapure water (Pureau, AU), and fixed for all measurements during the session [camera level 10, slider shutter 696, slider gain 73, detection threshold 5]. All uEV samples were analysed on 5 captures of 60 seconds. Sample dilution was initiated at 1/500, while
alternative dilutions were applied to obtain the recommended number of particles (50-100) per image.

- TMT labelled LC-MS/MS

  - Sample preparation and digestion

  Peptides for LC-MS/MS were generated using filter-aided sample preparation. In short, uEV samples were centrifuged at 16000×g for 1 h at 4°C and supernatants assayed for protein concentration using the Pierce BCA Protein Assay Kit (Thermo Scientific, IL, USA). 10 μg of individual exosome proteins were loaded onto a Vivacon-30 kDa spin column (Sartorius, Goettingen, Germany) by centrifugation at 16000×g. Spin columns were washed three times with UA buffer containing 8 M urea (Thermofisher, IL, USA) and 100 mM triethylammonium bicarbonate (Fisher Scientific, Leicestershire, UK). The proteins were then reduced using 50 mM of dithiothreitol (Thermofisher, IL, USA) for 1 h at 56 °C and alkylated with 50 mM 2-chloroacetamide for 20 minutes in the dark at room temperature, with centrifugation after each addition to remove excessive reagents. The spin columns were then washed one more time with UA buffer before adding 40 μl of Lys-C solution (FUJIFILM Wako, Osaka, Japan) (enzyme: protein = 1:50, dissolved in UA buffer). After 3h incubation at 37°C, 400 μl of trypsin solution (Promega, WI, USA) (enzyme: protein = 1:25, dissolved in 100 mM triethylammonium bicarbonate) was added, and the spin column was incubated at 37°C overnight. Peptides were collected by centrifuging the spin column in a new collection tube. Peptide concentration was measured using the Pierce Fluorometric Peptide Assay Kit (Thermofisher, IL, USA) according to the manufacturer’s protocol.

  - TMT labelling and fractionation
Peptides were labelled with TMT 10plex isobaric labeling reagents (Thermofisher, IL, USA). In brief, 0.4mg of each TMT tag (127N, 127C, 128N, 128C, 129N, 129C, 130N and 130C) was dissolved in 164μl acetonitrile, before adding to 400μl of peptide solution containing 6.4 μg peptides (Supplemental Table 2). A universal control channel containing an equal peptide amount from each sample was used and labelled with TMT tag 126 for comparisons between samples. After one hour at room temperature, individual labelling reactions were quenched by addition of 32 μl of 5% hydroxylamine. Individually labelled samples were combined and vacuum-dried in a SpeedVac, before fractionation using a Pierce high pH RP-fractionation kit (Thermofisher, IL, USA) according to the manufacturer’s protocol. In total three sets of TMT labelling were performed. Nine labelling channels were used in each set, with four patients (basal and post) occupying eight channels and one channel for the universal control.

- **LC-MS/MS analysis**

The TMT labelled samples were analyzed by nano liquid chromatography (nLC, EASY LC-1200, Thermo Fisher) coupled to a MS/MS system (Q Exactive Plus, Thermo Scientific) through an EASY-Spray nano-electrospray ion source (Thermo Scientific). A pre-column (Acclaim®PepMap 100, 75 μm x 2 cm, C18, 3 μm, 100 Å, Thermo Scientific) was used to trap peptides and an analytical column (EASY-Spray Column, PepMap, 75 μm x 25 cm, C18, 3 μm, 100 Å, Thermo Scientific) was used to separate peptides. For nLC separation, buffer A was 100% H2O/0.1% formic acid and buffer B was 80% ACN/0.1% formic acid. A linear gradient from 5% to 22% buffer B for 40 min, and then from 22% to 35% for 20 min was used for peptide separation. Precursor scans were performed at scan range of 300-1,600, a resolution of 70,000, maximum injection time of 100 ms and automatic gain
control of $3 \times 10^6$. MS/MS scans were up to 10 data-dependent acquisition scans performed at a resolution of 35,000, maximum injection time of 100 ms and automatic gain control of $1 \times 10^5$. Isolation window was set at 2 Da. Higher-energy-collisional-dissociation normalized collision energy was set at 33%. Fixed first mass was set at 110. Dynamic exclusion was set at 30 s. Rejection of precursor ions with charge state +1 and above +8 was employed.

- LC-MS/MS data analysis

LC-MS/MS raw files belonging to one TMT set were searched together against a UniProt human protein database (downloaded on 25th January 2019) using both the Sequest and Mascot algorithms through Proteome Discoverer software (v2.3). Quantification of peptides and proteins was done through the reporter ion quantification module of Proteome Discoverer, with corrections for TMT isotopic interferences enabled. Basal cursor mass tolerance was set as 10 ppm and fragment mass tolerance was set as 0.02 Da, and a maximum of 2 missed cleavage sites. Carbamidomethylation of cysteine was set as a static modification. Protein N-terminal acetylation, methionine oxidation, TMT labelling of peptide N-terminus and lysine were set as variable modifications. Peptide false discovery rate was calculated by Percolator and confined to $\leq 1\%$. Protein ratios obtained with the aid of the universal control channel TMT126 were log-transformed for paired $t$-test to determine the proteins that showed significant changes ($p<0.05$ with 95% confidence interval) post SSST. Differentially expressed protein (DEP) was identified as that with $p<0.05$, FDR$<0.1$ and fold change of $\geq 1.20$ or $\leq 0.83$.

- Immunoblotting validation
Defrosted uEVs were mixed thoroughly before 10 min centrifugation at 15000×g at 4°C to pellet insoluble residues. The supernatant was collected and total protein concentration was measured using a spectrophotometer (Thermo Scientific Nanodrop Lite). A uEV standard pool containing resuspension of mixed uEVs isolated from a large amount of urine collected from healthy normotensive volunteers between 9-11AM on multiple days was used as a universal control for normalisation of performance errors across all blots. uEVs were treated with 5x Laemmli sample buffer (1:4, v:v) and incubated at 60°C for 10 min before sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Twenty µg of each sample were loaded and separated on 8% poly-acrylamide mini gels, and were transferred to polyvinyl difluoride membranes (Bio-Rad) under 1.3A and 21V for 26 mins on a Bio-rad Turbo transferring system. Each blot was duplicated for proteins with similar size. Blots were then blocked with 5% BSA (A3858, Sigma) in Tris-Buffered saline (TBS), followed by overnight incubation with primary antibodies including: anti-AQP2 (1/1000; SPC-503, StressMarq Biosciences), anti-NCC (1/1000; AB3553, Merck Millipore), anti-pNCC (pT53/pT58; 1/1000), anti-ALIX (1/2000; ABC40, Merck Millipore), and anti-TSG101 (1/2000; MASBC649, Merck Millipore). AQP2 was measured by bands detected at 35-50 kDa (glycosylated) and at 29 kDa (unglycosylated protein); NCC and pNCC were measured by the dominant band around150 kDa despite the observation of dimerisation; uEV markers ALIX and TSG101 were measured by the dominant bands at 96 kDa and 45 kDa respectively. HRP conjugated goat anti-rabbit IgG antibody (12-348, Merck Millipore) was used as the secondary antibody at 1/20,000 for luminol-based enhanced chemiluminescence (1705061, Bio-rad), respectively before image capture using a Bio-
Rad ChemiDoc XRS+ Imager on configure signal accumulation mode with Image Lab software.

- Bioinformatic analyses

Calculations were processed with R. Overlap analyses were performed using Vesiclepedia and ExoCarta protein databases to compare the MS dataset with other human urine studies and to identify EV-enriched proteins. A rat renal transporter protein database (https://hpcwebapps.cit.nih.gov/ESBL/Database/NephronRNAsEq/Transporters_and_Channels.html) was used to identify renal transmembrane proteins. Gene ontology analysis was performed by ClueGO plugin (v2.5.5) in the Cytoscape environment (v3.7.2), and gene lists corresponding to 878 DEPs were used as input. GO terms were updated on 11th June 2020. Evidence level was set at “All without IEA (Inferred from Electronic Annotation)”. GO term fusion was enabled, and only pathways with $p \leq 0.01$ are shown. All other parameters were left at default.
Figure S1. Flow diagram of report numbers of individuals at each stage of study.

HTN patients with raised ARRs admitted for SSST were invited  
n= 44

All consented and completed SSST  
n= 44

Data available for analyses:
- Routine biochemistry  
n= 44
- Routine blood pressure  
n= 44
- Plasma Cl\(^{-}\) and HCO\(_3\)^{-}  
n= 41
- Plasma copeptin  
n= 33
- Spot urine creatinine  
n= 39
- uEV measured by MS  
n= 13
- uEV measured by WB  
n= 31
  - NCC  
n= 31
  - pNCC  
n= 31
  - AQP2  
n= 19
  - ALIX  
n= 31
  - TSG101  
n= 27
Figure S2. A heat map clustering of 294 differentially expressed proteins during SSST between PA and LRH subjects.
### Figure S3. Functional enrichment analyses of the 878 DEPs.

| Category                        | Biological process | Cellular component | Molecular function |
|--------------------------------|--------------------|--------------------|--------------------|
| Up-regulated DEPs              | 22.03              | 13.46              | 1.54               |
| Down-regulated DEPs            | 8.62               | 9.44               | 5.88               |

| Biological process          | Cellular component | Molecular function |
|-----------------------------|--------------------|--------------------|
| Up-regulated DEPs           | 21.65              | 0.15               |
| Down-regulated DEPs         | 9.44               | 0.88               |

| Biological process                          | Cellular component                          | Molecular function                      |
|---------------------------------------------|---------------------------------------------|------------------------------------------|
| Up-regulated DEPs                           | 1.72                                         | 5.88                                     |
| Down-regulated DEPs                         | 1.67                                         |                                          |

| Biological process                          | Cellular component                          | Molecular function                      |
|---------------------------------------------|---------------------------------------------|------------------------------------------|
| Up-regulated DEPs                           | 1.72                                         | 5.88                                     |
| Down-regulated DEPs                         | 1.67                                         |                                          |
Figure S4. Correlations between biochemical parameters and the 12 differentially expressed renal transmembrane proteins.
Figure S5. Immunoblots of analysed proteins.
| Participant#20 | Ctrl | Basal1 | Basal2 | Post |
|---------------|------|--------|--------|------|
| NCC-          |      |        |        |      |
| pNCC-         |      |        |        |      |
| ALIX-         |      |        |        |      |
| TSG101-       |      |        |        |      |

| Participant#21 | Ctrl | Basal1 | Basal2 | Post |
|----------------|------|--------|--------|------|
| NCC-           |      |        |        |      |
| pNCC-          |      |        |        |      |
| ALIX-          |      |        |        |      |
| TSG101-        |      |        |        |      |

| Participant#22 | Ctrl | Basal1 | Basal2 | Post |
|----------------|------|--------|--------|------|
| NCC-           |      |        |        |      |
| pNCC-          |      |        |        |      |
| ALIX-          |      |        |        |      |

| Participant#23 | Ctrl | Basal | Post |
|----------------|------|-------|------|
| NCC-           |      |       |      |
| pNCC-          |      |       |      |
| AQP2-          |      |       |      |
| ALIX-          |      |       |      |
| TSG101-        |      |       |      |

| Participant#24 | Ctrl | Basal | Post |
|----------------|------|-------|------|
| NCC-           |      |       |      |
| pNCC-          |      |       |      |
| AQP2-          |      |       |      |
| ALIX-          |      |       |      |
| TSG101-        |      |       |      |
Figure S6. Correlations between biochemical parameters and NCC, pNCC and AQP2.
**Figure S7.** Boxplots of changes in the total contents in the urine during SSST. PA, primary aldosteronism; LRH, low renin essential hypertension; Rel. Ab*Vol, protein relative abundance multiplying total urine volume.
Figure S8. Correlations between EV markers (quantified by immunoblotting [WB] and mass spectrometry [MS]) and spot urine creatinine.
Table S1. Participants’ clinical features and anti-hypertensive drugs at baseline.

| Patient No. | Sex | Age at SSST | BMI (kg/m²) | Dx     | SBP/DBP (mmHg/mmHg) | ADX | eGFR (ml/min) | Plasma creatinine (μmol/L) | Baseline plasma [K⁺] (mmol/L) | Baseline plasma ARR (pmol/mU) | Anti-HTN drugs without affecting RAS (daily) |
|-------------|-----|-------------|-------------|--------|---------------------|-----|---------------|-------------------------|-------------------------------|--------------------------------|----------------------------------|
|             |     |             |             |        |                     |     |               |                         |                               |                                | No. of drugs | Moxo (mcg) | Praz (mg) | Vera (mg) | Hydra (mg) |
| Normal range|     |             | 18.5 - 24.9 | <140/90 in adults | >60 | 45/90             | 3.5-5.5 | 2-75          |
| 1           | F   | 41.6        | 42.2        | LRH    | 143/93              | Non | >90           | 56                      | 3.6                           | 69                             | 1 - -                    |
| 2           | F   | 54.9        | 29.7        | LRH    | 160/110             | Non | >90           | 57                      | 4.1                           | 61                             | 1 - -                    |
| 3           | F   | 46.2        | 31.1        | LRH    | 118/82              | Non | >90           | 63                      | 4.1                           | 78                             | 1 - -                    |
| 4           | F   | 59.2        | 29.9        | PA     | 159/79              | Non | >90           | 49                      | 3.3                           | 1810                           | 3 200 -                  |
| 5           | F   | 56.9        | 23.5        | PA     | 150/88              | Non | >90           | 61                      | 3.1                           | 401                            | 0 - -                    |
| 6           | F   | 45.0        | 47.0        | PA     | 138/95              | Non | >90           | 52                      | 4.3                           | 129                            | 1 - -                    |
| 7           | F   | 39.8        | 43.5        | PA     | 145/115             | Non | >90           | 73                      | 3.2                           | 178                            | 1 - -                    |
| 8           | M   | 50.5        | 28.7        | PA     | 163/103             | Non | 83            | 92                      | 4.2                           | 51                             | 2 - 1 90 -               |
| 9           | M   | 58.3        | 51.4        | PA     | 155/87              | Non | 81            | 90                      | 2.6                           | 275                            | 2 - -                    |
| 10          | M   | 37.5        | 30.1        | PA     | 138/105             | Non | 82            | 101                     | 3.8                           | 336                            | 2 - - 240 -              |
| 11          | M   | 42.2        | 26.2        | PA     | 147/103             | Non | 83            | 97                      | 3.7                           | 232                            | 3 - 200 -                |
| 12          | M   | 31.8        | 37.4        | PA     | 180/111             | Non | >90           | 69                      | 2.9                           | 211                            | 3 - 1 120 -              |
| 13          | F   | 65.2        | 28.5        | PA     | 149/100             | Non | 74            | 74                      | 3.5                           | 352                            | 2 - - 120 -              |
| 14          | M   | 48.9        | 28.2        | PA     | 118/70              | Non | >90           | 78                      | 4.3                           | 65                             | 1 - -                    |
| 15          | F   | 69.9        | 27.9        | LRH    | 146/69              | Non | 90            | 60                      | 3.7                           | 276                            | 1 - 1 -                  |
| 16          | M   | 50.7        | 32.1        | PA     | 160/89              | Non | >90           | 76                      | 3.4                           | 121                            | 3 - 10 240 -             |
| 17          | F   | 39.3        | 27.7        | LRH    | 158/72              | Non | >90           | 43                      | 3.2                           | 54                             | 2 600 -                  |
| 18          | M   | 50.7        | 31.5        | PA     | 138/68              | Non | 75            | 100                     | 3.6                           | 166                            | 1 - - 90 -               |
| 19          | F   | 36.0        | 30.2        | LRH    | 154/100             | Non | >90           | 53                      | 3.8                           | 47                             | 0 - -                    |
| 20          | F   | 57.2        | 26.9        | PA     | 140/102             | Non | >90           | 62                      | 3.5                           | 109                            | 1 - -                    |
| 21          | F   | 67.8        | 29.6        | LRH    | 163/85              | Non | >90           | 45                      | 4.0                           | 212                            | 2 - - 120 -              |
| 22          | F   | 51.4        | 24.9        | PA     | 125/68              | Non | >90           | 59                      | 3.9                           | 95                             | 2 - - 240 -              |
| No. | Sex | Age | BMI  | Dx       | SBP      | DBP      | ARR | eGFR | Plasma [K+] | Plasma [K+] | SBP      | DBP      | ADX | RAS | Anti-HTN | Moxo | Praz | Vera | Hydra | Female/Male | PA | LRH | Primary Aldosteronism | SBP      | DBP      |
|-----|-----|-----|------|----------|----------|----------|-----|------|-------------|-------------|----------|----------|------|-----|----------|------|-----|------|-------|-----------|----|-----|-----------------------|----------|----------|
| 23  | F   | 51.6| 33.4 | LRH      | 149/83   | >90      | 56  | 4.4  | 41          | 2           | -        | -        | 200 | -  | -        | 240 | 100 |      |       | 180       |    |     | LRH, low renin essential hypertension | 240      | 100      |
| 24  | F   | 35.7| 32.8 | LRH      | 132/80   | >90      | 59  | 4.3  | 32          | 1           | -        | -        | 180 | -  | -        | 180 | -  |      |       | -         |    |     | LRH, low renin essential hypertension | 180      | -        |
| 25  | F   | 62.5| 30.0 | PA       | 110/65   | >90      | 65  | 4.3  | 128         | 1           | -        | -        | 180 | -  | -        | 180 | -  |      |       | -         |    |     | LRH, low renin essential hypertension | 180      | -        |
| 26  | M   | 38.6| 39.3 | PA       | 144/100  | >90      | 65  | 3.4  | 335         | 2           | 200      | 120      | 120 | -  | -        | 120 | -  |      |       | -         |    |     | LRH, low renin essential hypertension | 120      | -        |
| 27  | F   | 57.5| 24.1 | PA       | 140/80   | >90      | 65  | 3.8  | 301         | 0           | -        | -        | 180 | -  | -        | 180 | -  |      |       | -         |    |     | LRH, low renin essential hypertension | 180      | -        |
| 28  | F   | 42.8| 23.1 | LRH      | 125/100  | >90      | 75  | 3.8  | 144         | 2           | 400      | 240      | 240 | -  | -        | 240 | 75 |      |       | -         |    |     | LRH, low renin essential hypertension | 240      | 75       |
| 29  | F   | 69.5| 31.8 | PA       | 170/88   | >90      | 52  | 3.9  | 134         | 2           | -        | -        | 180 | -  | -        | 180 | -  |      |       | 240       |    |     | LRH, low renin essential hypertension | 240      | 75       |
| 30  | M   | 44.0| 28.5 | PA       | 156/106  | >90      | 78  | 3.5  | 609         | 4           | 400      | 4.5      | 480 | 150| 37.5      | 240 | 75 |      |       | -         |    |     | LRH, low renin essential hypertension | 240      | 75       |
| 31  | M   | 64.5| 35.8 | PA       | 138/74   | >90      | 73  | 3.8  | 134         | 2           | -        | -        | 180 | -  | -        | 180 | -  |      |       | 240       |    |     | LRH, low renin essential hypertension | 240      | 75       |
| 32  | F   | 64.7| 28.0 | PA       | 168/80   | >90      | 55  | 3.7  | 49          | 2           | -        | -        | 240 | 50 |         | 240 | 50 |      |       | -         |    |     | LRH, low renin essential hypertension | 240      | 50       |
| 33  | M   | 65.4| 31.6 | PA       | 166/102  | >90      | 73  | 3.9  | 130         | 3           | -        | 1.0      | 240 | 75| 37.5      | 240 | 75 |      |       | -         |    |     | LRH, low renin essential hypertension | 240      | 75       |
| 34  | F   | 55.3| 31.3 | PA       | 163/101  | >90      | 46  | 3.9  | 88          | 0           | -        | -        | 180 | -  | -        | 180 | -  |      |       | -         |    |     | LRH, low renin essential hypertension | 180      | -        |
| 35  | F   | 52.9| 29.4 | PA       | 122/80   | >90      | 52  | 3.8  | 243         | 1           | -        | -        | 360 | -  |         | 360 | -  |      |       | -         |    |     | LRH, low renin essential hypertension | 360      | -        |
| 36  | M   | 68.1| 34.0 | PA       | 142/92   | >90      | 87  | 3.7  | 136         | 2           | -        | -        | 240 | 50| 37.5      | 240 | 50 |      |       | -         |    |     | LRH, low renin essential hypertension | 240      | 50       |
| 37  | M   | 66.2| 26.0 | PA       | 161/58   | >90      | 68  | 2.8  | 143         | 3           | 200      | 4.0      | 240 | -  | -        | 240 | -  |      |       | -         |    |     | LRH, low renin essential hypertension | 240      | -        |
| 38  | F   | 43.6| 41.8 | PA       | 118/82   | >90      | 57  | 4.1  | 51          | 1           | -        | -        | 240 | -  | -        | 240 | -  |      |       | -         |    |     | LRH, low renin essential hypertension | 240      | -        |
| 39  | F   | 53.6| 27.3 | PA       | 164/92   | >90      | 61  | 3.6  | 281         | 2           | -        | -        | 180 | 25|         | 180 | 25 |      |       | -         |    |     | LRH, low renin essential hypertension | 180      | 25       |
| 40  | F   | 45.1| 38.1 | PA       | 164/71   | >90      | 57  | 4.2  | 36          | 2           | 400      | 360      | 360 | -  | -        | 360 | -  |      |       | -         |    |     | LRH, low renin essential hypertension | 360      | -        |
| 41  | F   | 39.3| 25.6 | PA       | 134/84   | >90      | 77  | 4.0  | 98          | 0           | -        | -        | 180 | -  | -        | 180 | -  |      |       | -         |    |     | LRH, low renin essential hypertension | 180      | -        |
| 42  | F   | 51.7| 25.6 | PA       | 100/60   | >90      | 55  | 3.6  | 246         | 0           | -        | -        | 360 | -  |         | 360 | -  |      |       | -         |    |     | LRH, low renin essential hypertension | 360      | -        |
| 43  | M   | 38.5| 36.8 | PA       | 170/98   | >90      | 75  | 3.7  | 48          | 4           | 600      | 4        | 360 | 75|         | 360 | 75 |      |       | -         |    |     | LRH, low renin essential hypertension | 360      | 75       |
| 44  | F   | 70.4| 39.0 | PA       | 154/69   | >90      | 77  | 3.9  | 122         | 4           | 600      | 4        | 600 | 100|         | 600 | 100|      |       | -         |    |     | LRH, low renin essential hypertension | 600      | 100      |

No., number; SSST, seated saline suppression testing; BMI, body mass index; Dx, diagnosis of primary aldosteronism; SBP, systolic blood pressure; DBP, diastolic blood pressure; ADX, unilateral adrenalectomy; eGFR, estimated glomerular filtration rate; plasma [K⁺], plasma potassium concentration; ARR, aldosterone-to-renin ratio; anti-HTN, anti-hypertensive; RAS, renin angiotensin ii system; Moxo, Moxonidine; Praz, Prazosine; Vera, Verapamil; Hydra, Hydralazine; F, female; M, male; PA, primary aldosteronism; LRH, low renin essential hypertension.
Table S2. NTA measures of particle size and concentration of nine uEV samples.

| Sample No. | Dilution | Particle Size (nm) | Participle concentration (*10^9 particle/mL) | Particles/frame |
|------------|----------|--------------------|---------------------------------------------|-----------------|
|            |          | Mean               | Mode                                        |                 |
| 1          | 1:750    | 218.9 ± 1.6        | 142.8 ± 7.2                                 | 1.55 ± 0.074    | 78.6 ± 3.7 |
| 2          | 1:750    | 266.0 ± 3.1        | 171.4 ± 13.9                                | 1.65 ± 0.022    | 83.7 ± 1.1 |
| 3          | 1:500    | 222.8 ± 11.4       | 139.9 ± 7.0                                 | 0.99 ± 0.11     | 50.2 ± 5.6 |
| 4          | 1:500    | 230.6 ± 5.5        | 146.1 ± 2.5                                 | 1.38 ± 0.028    | 70.3 ± 1.4 |
| 5          | 1:250    | 227.9 ± 6.0        | 143.8 ± 4.8                                 | 1.29 ± 0.026    | 65.5 ± 1.3 |
| 6          | 1:250    | 341.3 ± 5.7        | 178.5 ± 3.6                                 | 1.37 ± 0.040    | 69.4 ± 2.0 |
| 7          | 1:750    | 341.7 ± 5.9        | 194.9 ± 6.7                                 | 1.37 ± 0.089    | 69.4 ±4.5 |
| 8          | 1:750    | 306.9 ± 8.6        | 179.6 ± 8.1                                 | 1.76 ± 0.063    | 89.2 ± 3.2 |
| 9          | 1:150    | 271.9 ± 4.1        | 163.3 ± 11.5                                | 1.93 ± 0.053    | 98.2 ± 2.7 |
| Abbreviations | Description |
|---------------|-------------|
| ALIX          | Apoptosis-Linked Gene 2-Interacting Protein X |
| AQP2          | Aquaporin 2 |
| ARR           | Aldosterone-to-Renin Ratio |
| CD9           | Tetraspanin CD9 |
| Cl-           | Chloride |
| DEP           | Differentially Expressed Protein |
| ESCRT         | The Endosomal Sorting Complexes Required for Transport |
| EV            | Extracellular Vesicles |
| FC            | Fold Change |
| FDR           | False Discovery Rate |
| GO            | Gene Ontology |
| HCO3-         | Bicarbonate |
| K+            | Potassium |
| KCl           | Potassium Chloride |
| LC-MS/MS      | Liquid Chromatography with Tandem Mass Spectrometry |
| LRH           | Low Renin Essential Hypertension |
| Na+           | Sodium |
| NaCl          | Sodium Chloride |
| NCC           | Sodium Chloride Cotransporter |
| NTA           | Nanoparticle Tracking Analysis |
| PA            | Primary Aldosteronism |
| pNCC          | Phosphorylated NCC |
| RAAS          | Renin Angiotensin Aldosterone System |
| SSSST         | Seated Saline Suppression Testing |
| TMT           | Tandem Mass Tag |
| TSG101        | Tumour Susceptibility Gene 101 |
| uEVs          | Urinary Extracellular Vesicles |