Increased Blood Pressure, Aldosterone Activity, and Regional Differences in Renal ENaC Protein During Vasopressin-Escape

Short title: MAP, Aldosterone, and ENaC in vasopressin escape

by

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ABSTRACT

The syndrome of inappropriate anti-diuretic hormone (SIADH) is associated with water retention and hyponatremia. The kidney adapts via a transient natriuresis and persistent diuresis, i.e., vasopressin escape. Previously, we showed an increase in the whole kidney abundance of aldosterone-sensitive proteins, the α- and γ- (70-kDa band) subunits of the epithelial sodium channel (ENaC) and the thiazide-sensitive Na-Cl cotransporter (NCC) in our rat model of SIADH. Here we examine mean arterial pressure (MAP) via radiotelemetry, aldosterone activity, and cortical versus medullary ENaC subunit and 11-β-hydroxysteroid dehydrogenase type 2 (11-β-HSD-2) protein abundances in escape. Eighteen male, Sprague-Dawley rats (300 g) were sham operated (n = 6) or infused with dDAVP (n = 12, a V2-receptor selective analogue of AVP). After 4 days, half of the rats receiving dDAVP were switched to a liquid diet, i.e., water loaded (WL) for 5-7 additional days. The WL rats had a sustained increase in urine volume and blood pressure (122 versus 104 mm Hg, p <0.03, at 7 days). Urine and plasma aldosterone levels were increased in the WL group to 844 and 1658% of the dDAVP group, respectively. NCC and α- and γ-ENaC (70-kDa band) were significantly increased in the WL group (relative to dDAVP), only in the cortex. β- and γ-ENaC (85-kDa band) were significantly increased by dDAVP relative to control, in cortex and medulla. 11-β-HSD-2 was increased by dDAVP in the cortex and not significantly affected by water loading. These changes may serve to attenuate sodium losses and ameliorate hyponatremia in vasopressin escape.

Keywords: hyponatremia, SIADH, pressure natriuresis, renin, 11-β-hydroxysteroid dehydrogenase
INTRODUCTION

Hyponatremia is one of the most common, and potentially serious, clinical disorders observed in hospitalized patients (34). One cause of hyponatremia is the syndrome of inappropriate antidiuretic hormone secretion (SIADH). With this disorder, patients secrete abnormally high levels of vasopressin (ADH) relative to plasma osmolality, resulting in inappropriate free-water retention and a fall in serum [Na⁺]. This water-retentive phase is generally relieved by a physiological process known as “vasopressin escape”, in which both humans and animal models undergo a natriuresis followed closely by a diuresis of increasingly more dilute urine. The mechanisms and triggers of both the diuresis and the natriuresis are not fully understood, although increased renal arterial pressure appears essential for the process (16).

It is logical to assume that this natriuresis might contribute to a further reduction in serum Na⁺ levels unless adaptive compensatory mechanisms are invoked. Several reports (1, 6, 7, 22, 23, 30), going back decades have reported evidence of increased aldosterone activity, i.e., plasma levels, urine excretion, and urine K⁺ to Na⁺ ratios in hyponatremia. These results were surprising, because SIADH is often associated with volume expansion, which is known to decrease activation of the renin-angiotensin system thereby reducing angiotensin II stimulation of adrenal aldosterone secretion. Also some reports (5, 12, 14, 15) did suggest decreased rather than increased aldosterone secretion in SIADH in humans and animal models. Furthermore, the mechanisms proposed to underlie these increases in aldosterone activity in SIADH were not clear and hypotheses ranged from a direct effect of low Na⁺ on the adrenal to increased sensitivity of the adrenal to angiotensin II, ACTH, or K⁺.

Much has been determined in the last decade with regard to the regulation of aldosterone-responsive sodium transport proteins in the kidney. Aldosterone-sensitive NaCl transport has been demonstrated in the distal convoluted tubule via the thiazide-sensitive NaCl cotransporter
TSC or NCC) (33) and in the collecting duct via the amiloride-sensitive sodium channel, (ENaC) (17). Utilizing immunoblotting and immunohistochemistry, Kim et al. (19) found a marked increase in the protein abundance of the thiazide-sensitive Na-Cl cotransporter (TSC or NCC) with either aldosterone infusion or low-NaCl diet. NCC is expressed exclusively in the distal convoluted tubule in the cortex of the kidney. Likewise, Masilamani et al. (24) showed an increase in α-ENaC and the 70-kDa band associated with γ-ENaC in the collecting duct in these same animal models. Recently (12), we showed the same pattern of protein changes in whole kidney homogenates in our rat model of vasopressin escape. In those animals, we were unable to detect a significant increase in plasma aldosterone levels collected at the time of sacrifice. However, due to the striking nature of this pattern of changes in ENaC expression, and the fact that aldosterone regulation in SIADH has remained poorly understood, we elected to reevaluate this parameter. In this study we evaluated the regulation of aldosterone by measuring it both in the plasma and urine, as well as, the abundances of ENaC subunits, NCC, and the enzyme, 11-β-HSD-2 in the cortex, inner stripe of the outer medulla, and inner medulla of the kidney throughout escape from vasopressin-induced antidiuresis. A decrease in the abundance and activity of 11-β-HSD-2 could explain an aldosterone-like pattern of protein changes in the kidney. This enzyme is responsible for the conversion of corticosterone to cortisol in cells that express it. Thus, under normal conditions it “shields” the mineralocorticoid receptor, which can bind both mineralocorticoids, such as aldosterone, and glucocorticoids, such as corticosterone, from corticosterone which circulates in plasma at 100-fold higher concentrations.

We also utilized state-of-the-art radiotelemetry to continuously monitor blood pressure over the course of vasopressin escape. Previously (35) we found a slight increase in mean arterial pressure (MAP) in our rat model of SIADH measured at the end of the study period. In this study, we determined the time-course of blood pressure changes and correlated them to changes in aldosterone activity and the natriuresis and diuresis of vasopressin escape.
MATERIALS AND METHODS

Animals and study design. Three studies were performed. For all studies, male, Sprague-Dawley rats, approximately 275 g, were obtained from Taconic Farms, Germantown, MD. In the first, 18 rats were divided into 3 treatment groups (n = 6/group): 1) control, 2) dDAVP-infused, 3) dDAVP-infused plus feeding of a liquid diet. All rats were anesthetized with isoflurane (IsoFlo®, Abbot Laboratories, North Chicago, IL) prior to subcutaneous osmotic mini-pump implantation (Alzet model 2002, Cupertino, CA). Desmopressin (dDAVP), 1-deamino-[8-D-arginine]-vasopressin (Aventis Pharmaceuticals, Bridgewater, NJ), a selective vasopressin V2 receptor agonist, was infused into rats in groups 2 and 3 at a rate of 5 ng/hour, as previously described (9, 12, 13). At this time, all rats were fed a dry, pelleted AIN-76 formulation diet (BioServe, Frenchtown, NJ). After 4 days, rats in group 3 were switched to liquid AIN-76 diet containing a high amount of water (80% by weight) or continued on the dry, pelleted diet and water ad libitum for 5 additional days. Rats were housed continuously in Nalgene® metabolic cages (Harvard Apparatus, Holliston, MA) to facilitate urine collection. Finally, all rats were euthanized by decapitation and both heparinized and K⁺-EDTA blood was collected for plasma hormone analyses. The right kidney was rapidly removed and frozen at –80°C for later dissection and immunoblotting analyses (see below).

Study 2 had the same design as Study 1, except n = 4/treatment. At the end of the study, rats were anesthetized with isoflurane and the left kidney was perfusion fixed with 2% paraformaldehyde following phosphate-buffered saline to remove blood.

In Study 3, twelve male Sprague-Dawley rats were implanted with radiotelemetry blood pressure transmitters (Data Sciences International, St. Paul, MN). Briefly, under isoflurane anesthesia, the pressure-sensitive tip of the fluid-filled catheter of the radiotransmitter was advanced into the aorta via an incision in the femoral artery. The body of the radiotransmitter
was secured in a pouch under the skin near the left hind limb. Blood pressure was measured for 10 seconds at 10-minute intervals for the entire study. After a 5 - 7 day recovery period, these rats were randomly assigned to one of two treatments (n = 6/group): 1) dDAVP infusion or 2) dDAVP infusion plus liquid diet (as above). Surgeries to implant minipumps were done as described above and then blood pressures were monitored for 7 additional days. Rats were euthanized by decapitation. All animals were maintained at all times under conditions and protocols approved by the Georgetown University Animal Care and Use Committee, an AAALAC (American Association for Accreditation of Laboratory Animal Care) approved facility.

**Urine and plasma analyses.** Urine was analyzed for sodium, potassium (ion-selective electrode system, EL-ISE electrolyte system, Beckman Instruments Inc. Brea, CA), osmolality, (freezing-point depression, The Advanced Osmometer® Model 3900, Advanced Instruments, Inc., Norwood, MA) and aldosterone [Coat-a-Count® radioimmunoassay (RIA) kit, Diagnostic Products Corporation, Los Angeles, CA]. Whole blood was centrifuged at 3000 rpm (Sorvall RT 6000 D, Sorvall, Newtown CT) at 4°C for 20 minutes to separate plasma. Plasma was analyzed for sodium, potassium, osmolality, aldosterone, renin activity (RIA, Diagnostic Products Corporation), and ACTH (RIA, DiaSorin, Stillwater, MN).

**Immunoblotting.** Whole cortex, outer, and inner medullary homogenates of the right kidney were prepared as previously described (11, 12). Initially, coomassie-stained “loading gels” were done in order to assess the quality of the protein by sharpness of the bands and to confirm equality of loading, as previously described (10, 12). For immunoblotting, 20 - 30 µg of protein from each sample was loaded into individual lanes of minigels of 7 or 10% polyacrylamide (precast, BioRad, Hercules, CA). Blots were probed with our own polyclonal antibodies against NCC, and the α-, β-, and γ-subunits of ENaC. Our rabbits were immunized using the same peptide sequences as those used by Knepper and associates (19, 21) and our purified antibodies recognized the same molecular weight bands on immunoblots. The 11-β-
HSD-2 antibody was a sheep polyclonal obtained from Chemicon, (Temecula, CA).

**Immunohistochemistry for 11-β-HSD-2.** The left kidney from Study 2 was processed to paraffin and 5 μm sections were cut. Heat-induced target retrieval was performed using citrate buffer pH 6 (Zymed Laboratories, CA) to unmask antigenic sites. Endogenous peroxidase activity was removed by incubation with 2% H₂O₂ (DakoCytomation, Denmark) for 20 minutes. Tissues were incubated with 11-β-HSD-2 antibody (1:1000) overnight at 4°C. Sections were incubated with secondary antibody, rabbit anti-sheep IgG conjugated to horseradish peroxidase (KPL, Gaithersburg, MD), for one hour and then with ABC solution of the Vectastain Elite Peroxidase Kit (Vector Labs, CA) for one hour at room temperature. 3,–3'-diaminobenzidine tetrachloride dihydrate (DAB) was applied for 10 minutes and the tissue was counterstained with Mayer’s hematoxylin to allow anatomical definition. A positive reaction was identified as a brown stain in the cytoplasm or a dark brown/black nuclear stain as a result of superimposition of the DAB reaction and the blue counterstain.

**Statistics.** Data were evaluated by Sigma Stat (Chicago, IL). One-way analysis of variance followed by Tukey’s multiple comparison’s test (when significant differences were determined between groups) was used to analyze data from Study 1. For Study 3, unpaired t-test was used to evaluate differences between the two treatments in the daily mean arterial blood pressures (MAPs). To determine a daily average MAP for each rat, each day, all blood pressure readings for that day were averaged, i.e., 144 measurements/day. The Data Sciences software program, Dataquest® was set to “clip” data, that fell out of the range 50 < reading < 200 mm Hg, as being spurious data.

**RESULTS**

**Verifying Escape.** Urine volume is plotted in Figure 1A. Similar to previous studies (9, 12, 13), rats began to “escape” from the anti-diuresis of dDAVP, by day 2 or 3 after initiation
water loading. Likewise, urine osmolality (1B) was significantly reduced in the rats undergoing escape, relative to dDAVP-treated rats, in this study by day 2 of water loading. The marked natriuresis of vasopressin escape began on day one (Figure 2) and peaked by day 2, as observed previously (12).

**Aldosterone Activity.** In Figure 3A, we have plotted the urine potassium to sodium ratio as an index of aldosterone activity in the kidney. This ratio should increase, independent of feed intake, when aldosterone activity is high in the kidney. In the first 2 days of water-loading this ratio plunged in the water-loaded group, (during the period of natriuresis). However, starting on day 3 this ratio reversed and showed a sharp, upward slope of increase over the next 3 days, indicating increasing relative aldosterone activity. Figure 3B shows urine aldosterone excretion in these rats. Urine aldosterone was low, and not different among all treatments until 3 days after the beginning of water loading, when it began to markedly increase in the water-loaded rats to a level of 844% of that in the dDAVP-treated rats by day 5.

**Plasma Biochemistry.** As expected, rats receiving the water load were hypoosmotic and hyponatremic (Table 1). Plasma aldosterone levels, by day 5, were significantly increased by the water load to 1658%, whereas, renin activity was markedly suppressed to 4% of the dDAVP-treated level by day 5. No significant differences were observed for plasma ACTH levels among the groups.

**Increased Mean Arterial Blood Pressure (MAP) in Rats Undergoing Escape.** In Figure 4, the daily treatment means of the MAP are plotted for the rats in Study 2. MAP rose slightly in both groups of rats after they began the dDAVP infusion. However, divergence between treatment means for MAP began at 2 days of water loading, where MAP for the water-loaded group continued to rise for 3 additional days (to day 5). At that point, it plateaued at an approximately 20 mm Hg elevation relative to the dDAVP-treatment alone.
NCC and Differential Regional Regulation of ENaC Subunit Abundances. In Figure 5, example immunoblots derived from cortex, outer, or inner medullary homogenates of the rats in Study 1 probed with antibodies against NCC (cortex only), α-, β-, and γ-ENaC, are shown. NCC was not examined in the inner and outer medulla because it is expressed exclusively in the distal convoluted tubule found only in cortical homogenates. Table 2 provides a densitometry summary of these data and statistics. Similar to our previous findings using whole kidney homogenates (12), we found a mineralocorticoid-like pattern of protein changes in the cortex. That is, there was an increase in abundance of NCC, α-ENaC and the 70-kDa band of γ-ENaC (broad band region) with water loading. In contrast, this pattern of changes was different in the outer or inner medulla. α-ENaC was actually decreased in abundance by dDAVP infusion in the inner and outer medulla, and was not affected at all by the water-loading. The 70-KDa band of γ-ENaC was increased by dDAVP in the medulla irrespective of whether or not the animals were water-loaded. On the other hand, β-ENaC was increased by dDAVP infusion in all three regions. However, in the CTX, it was increased more in the dDAVP + WL rats relative to the dDAVP alone treated group. Similarly, the upper (85-kDa band) was strongly upregulated by dDAVP infusion in all three kidney regions. Finally, in the cortex, our anti-γ-ENaC antibody also recognized a band around 60 kDa, the nature of which is not known. This band did not appear to be regulated in this study.

Regulation of 11-β-Hydroxysteroid Dehydrogenase-2 (11-β-HSD-2) Protein. A decrease in the activity of the enzyme 11-β-HSD-2, would predict an increase in both blood pressure and the abundance of proteins regulated via activity at the mineralocorticoid receptor. In Figure 6, we show immunoblots of cortex, outer, and inner medullary homogenates probed with 11-β-HSD-2 antibody. In agreement with Brooks et al. (3), 11-β-HSD-2 tended to be increased with dDAVP, but it did not appear to be decreased in the water-loaded rats relative to either of the other two treatment groups. Furthermore, as shown previously (2), 11-β-HSD-2
was expressed exclusively in the distal tubule, specifically, the distal convoluted tubule and the collecting ducts (Figure 7). Labeling was strongest in the cortex and outer medulla (including the inner stripe) and diminished in the inner medullary papillary tip. No clear treatment differences could be ascertained by immunohistochemistry.

DISCUSSION

The interplay between sodium and water balance in the body is quite intricate. Differential regulation of transport along the renal tubule by vasopressin and aldosterone, respectively allows for selective reabsorption of sodium or water, as needed. SIADH, characterized by hyponatremia, is primarily the result of dysregulated water balance. Sodium, in the plasma and the cells, is diluted as water is retained. However, concurrent with water retention, a marked transient natriuresis occurs. This is likely an adaptive response of the body to reduce plasma volume as increased arterial pressure is detected by baroreceptors. In order to prevent excessive sodium losses due to natriuresis it is not unreasonable to predict adaptive sodium-retentive mechanisms may come into play in order to reestablish sodium balance. One possibility to accomplish this is upregulation of aldosterone production and excretion and its regulated proteins.

What is the stimulus for hyperaldosteronism in vasopressin escape? Although, not considered a classical stimulus, as high extracellular [K⁺], angiotensin II, and ACTH are recognized to be, several studies (1, 6, 7, 23, 28, 30, 37) suggest that hyponatremia is associated with direct or indirect stimulation of adrenal aldosterone synthesis. Balla et al. (1) used peritoneal dialysis with a 5% glucose solution in dexamethasone-pretreated rats to produce hyponatremia. They found an increase in plasma aldosterone levels when sodium levels dropped below 132 mM. Furthermore, Decaux and associates (7) have demonstrated a corticosteroid-dependent nature of the hyperaldosteronism. They showed that panhypopituitaric
rats treated with water and dDAVP do not develop hyperaldosteronism, as do the same rats treated with corticosteroids. We found no significant differences in the level of corticosterone (previous study) (12) or ACTH (Table 1) between treatment groups, however, whether or not their presence at any level was required to produce hyperaldosteronism was not tested.

A few groups (23, 28, 30, 37) have examined potential cellular signaling mechanisms for the hyperaldosteronism. Makara et al. (23) demonstrated increased sensitivity of rat adrenal glomerulosa cells to K⁺ when the cells were incubated in hypoosmotic conditions (250 mOsm) as demonstrated by increased Ca⁺ currents and aldosterone secretion. Schneider and associates (28, 30, 37) have reported osmolality, independently of sodium concentration, affects basal, K⁺- and angiotensin-stimulated aldosterone secretion in bovine or canine glomerulosa cells. They suggest that aldosterone secretion is likely mediated via hypoosmotic activation of voltage-dependent or stretch-activated calcium channels (37).

There are several possible reasons why the effect of hyponatremia on aldosterone secretion is not well recognized. First, there have been conflicting reports as to whether or not it really occurs (5, 12, 14, 15). Cogan et al. (5) and Fichman et al. (14, 15) have shown depressed or normal aldosterone levels, respectively in patients with SIADH. Second, hyponatremia is considered a pathophysiological state, and the classical stimuli are generally considered to be the major contributors to aldosterone stimulation under normal physiological conditions. Third, there appears to be a delay in the rise in aldosterone relative to the development of hyponatremia, making it unclear what the stimulus for secretion truly is (22). As early as 1965, Knochel and associates (22) reported a delayed rise in aldosterone excretion in a patient with SIADH administered a water load. We speculate, as do others (22), that the “delay” in the appearance of hyperaldosteronism relative to the rapid development of hyponatremia may be due to the competition between factors that decrease aldosterone, i.e., volume expansion,
and factors that increase it, i.e., hyponatremia. This might also explain conflicting findings of many groups including ourselves (12).

In these studies we show a gradual, but sustained increase in MAP (10-20 mm Hg) in rats infused with dDAVP after they were switched to a “liquid diet”. Blood pressure begins to increase one to two days after starting the liquid diet and plateaus by day 4. The mechanism for the increase in blood pressure cannot be determined here. SIADH is not commonly associated with hypertension in humans (16, 27). However, because these patients are generally quite ill to begin with, mild elevations in blood pressure might be overlooked. Case reports of elevated blood pressure in association with SIADH do exist (18, 26, 31), however, it is unclear what is cause and effect in these complex disorders. Several years ago (35), we showed an elevation in blood pressure of a similar magnitude in our rat vasopressin-escape model. However, in that study blood pressure was monitored for only 120 minutes at the completion of the study, in conscious animals via an indwelling aortic catheter, as opposed to continuously via telemetry from the outset of the study. In dogs using a servo-controlled renal arterial pressure clamped model, Hall and associates (16) demonstrated that a rise in renal arterial pressure was necessary to elicit the diuresis and natriuresis of vasopressin escape.

It seems plausible that high aldosterone activity was the cause of the increase in the protein abundances of α-ENaC, NCC, and the 70-kDa band of γ-ENaC, because this is the pattern that is produced by aldosterone infusion or feeding of a low-NaCl diet to rats (19, 24), although we did not directly test that assumption here. In this study we also found an increase in β-ENaC in the cortex of the water-loaded rats relative to both other groups. In our previous studies (12) we did not observe this difference, however, in those studies we examined whole kidney rather than cortex homogenates and were using a different polyclonal antibody. Curiously, we find that regulation ENaC subunit abundance is different in the outer and inner medulla relative to the cortex. Although regulation of the ENaC subunit abundances by
aldosterone, angiotensin II, and vasopressin in the whole kidney or the cortex have been well
described, little has been done examining regulation in the outer and inner medullary portion of
the collecting duct. This may partly relate to the fact that the presence of ENaC subunit
expression in the inner medullary collecting duct (IMCD) is controversial (4, 8, 32, 36), with
some reporting little or no ENaC mRNA in these cells (8, 32). Nevertheless, recently, Nielsen
and associates have examined the regulation of ENaC subunit protein abundances in all three
regions of the CD. They have reported differential regional regulation in rat models of lithium
toxicity (25) and puromycin aminonucleoside (PAN) nephrotic syndrome (20). With lithium
toxicity, β- and γ-ENaC were down-regulated in protein abundance in cortex and outer medulla,
but not in inner medulla or connecting tubule. They suggested, as one possible explanation, a
cell-specific resistance to aldosterone and vasopressin in the cortical and outer medullary CD.
Differences in sensitivity may relate to differences in expression patterns of both the
mineralocorticoid receptors (MR) and 11-β-dehydroxysteroid dehydrogenase subtype II (11-β-
HSD-2). We have confirmed others (2, 29) findings of decreased inner medullary relative to
outer medullary and cortical abundance of 11-β-HSD-2, which could explain increase sensitivity
to changes in circulating aldosterone levels in the cortex relative to the medulla. However, Wolf
et al. (38) have reported an increase in α-ENaC mRNA in cortex, outer, and inner medulla of
rats fed a low-NaCl diet, suggesting aldosterone-sensitivity of at least this subunit in the inner
medulla. We did see a trend for an increase in abundance of 11-β-HSD-2 with dDAVP infusion,
as Brooks et al. (3) have reported, but did not observe any relative down-regulation of this
abundance with water-loading, that might explain the mineralocorticoid-like pattern of protein
changes present only in the cortex. However, it is possible that activity of this enzyme however
is diminished without a change in abundance. Additional studies will be needed to understand
the mechanisms underlying differential regional or cellular regulation of ENaC subunits during
physiological and pathophysiological states.
Overall these studies show clearly an increase in plasma and urine aldosterone and MAP in rats undergoing vasopressin escape. Renal distal tubule proteins known to be regulated by aldosterone are also strongly upregulated in this model. These adaptive changes may facilitate the escape process, while attenuating the severity of sodium losses.

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GRANTS

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REFERENCES


17. Hawk CT, Li L, and Schafer JA. AVP and aldosterone at physiological concentrations have synergistic effects on Na+ transport in rat CCD. Kidney Int Suppl 57: S35-41, 1996.


FIGURE LEGENDS

FIGURE 1. Urine volume and osmolality during vasopressin escape. A) Urine volume over the course of the experiment with water loading commencing on Day 0. Urine volume was significantly increased in the dDAVP plus water load group relative to the dDAVP/dry diet group by 3 days. B) Urine osmolality over the course of the experiment. Osmolality was significantly decreased by water-loading in the dAVP-treated rats relative to dDAVP-treatment alone, by day 2. Tukey’s multiple comparisons test was applied only when one-way ANOVA detected a significant (p < 0.05) difference among means. Letters were assigned based on the outcome of the Tukey’s multiple comparison’s test. Means with letters in common are not statistically different from each other.

FIGURE 2. Urine sodium excretion during vasopressin escape. dDAVP plus water loading lead to a marked natriuresis that peaked on day 2.

FIGURE 3. Urine potassium to sodium ratio and aldosterone. A) the ratio of potassium to sodium was measured as an index of renal aldosterone activity. This ratio was significantly suppressed in the early part of water loading (through day 2). However, after that there was a remarkable recovery indicating increasing sodium relative to potassium retention in the escaping rats as days progressed. B) Aldosterone excretion over the course of vasopressin escape. Urine aldosterone was low, and not different among groups until day 3, on which the water-loaded rats had increased excretion of aldosterone which continued to increase remarkably over the rest of the experiment. Tukey’s multiple comparisons test was applied only when one-way ANOVA detected a significant (p < 0.05) difference among means. Letters were assigned based on the outcome of the Tukey’s multiple comparison’s test. Means with letters in common are not statistically different from each other.
FIGURE 4. Mean arterial blood pressure during vasopressin escape (n = 6/group). Water-loading (with dDAVP) caused a rapid and sustained increase in MAP relative to dDAVP alone. *indicates a significant (p < 0.05) difference between the groups as determined by unpaired t-test.

FIGURE 5. Targeted proteomics of distal renal sodium transporters and channel subunits during vasopressin escape. Each lane of each blot is loaded with a different rat’s sample (n = 6/treatment) of whole homogenate of either cortex, outer, or inner medulla (as indicated). Within each blot, equal amounts of total protein were loaded in each lane and a coomassie-stained gel confirmed equality of loading for each sample set. Blots were probed with polyclonal antibodies against NCC, α-, β- or γ-ENaC. A summary of densitometry and statistics are provided in Table 2.

FIGURE 6. Immunoblotting of 11-β-HSD-2 protein in kidney regions during vasopressin escape. A. Each lane of each blot is loaded with a different rat’s sample (n = 6/treatment) of whole homogenate of either cortex, outer, or inner medulla (as indicated). Within each blot, equal amounts of total protein were loaded in each lane and a coomassie-stained gel confirmed equality of loading for each sample set. Blots were probed with the polyclonal antibody against 11-β-HSD-2. B. Summary of densitometry for cortex (top), outer medulla (middle) and inner medulla (bottom). One-way ANOVA followed by Tukey’s test revealed a significant increase in band density for dDAVP + WL versus control rats in cortex.

FIGURE 7. Immunoperoxidase-labeling of 11-β-HSD-2 in kidney during vasopressin escape. A- B- control rat; C-D- dDAVP-treated rat; E-F- dDAVP + WL-treated rat. A, C, E- low magnification (40X) images showing the strongest labeling in the cortex through outer medulla; The inner stripe of the outer medulla was labeled most intensely in the both groups of dDAVP-treated rats (C and E) versus the control (A); B, D, F- high magnification (200X) of the cortex showing labeling of collecting ducts and distal convoluted tubules.
Table 1: Plasma Physiology in Vasopressin Escape*

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<td>Aldosterone (nM)</td>
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<td>10.1 ± 1.8‌^A‌</td>
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*Plasma collected at sacrifice; mean ± sem †generated angiotensin I. ‡Within a row, the mean is significantly (p < 0.05) increased relative to means labeled with "B". ‡the mean is significantly decreased relative to means labeled with “A”. ‡AB the mean is not significantly different from means labeled with either “A” or “B”, as assessed by one-way ANOVA followed by Tukey’s multiple comparisons test.
Table 2: Densitometry Summary of Immunoblots*

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<td>94 ± 14^B</td>
<td>299 ± 14^A</td>
</tr>
<tr>
<td>α-ENaC</td>
<td>CTX</td>
<td>100 ± 5^B</td>
<td>104 ± 11^B</td>
<td>196 ± 11^A</td>
</tr>
<tr>
<td>α-ENaC</td>
<td>OM</td>
<td>100 ± 3^A</td>
<td>72 ± 3^B</td>
<td>72 ± 5^B</td>
</tr>
<tr>
<td>α-ENaC</td>
<td>IM</td>
<td>100 ± 5</td>
<td>90 ± 7</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>β-ENaC</td>
<td>CTX</td>
<td>100 ± 21^C</td>
<td>314 ± 35^B</td>
<td>545 ± 56^A</td>
</tr>
<tr>
<td>β-ENaC</td>
<td>OM</td>
<td>100 ± 15^B</td>
<td>212 ± 11^A</td>
<td>203 ± 26^A</td>
</tr>
<tr>
<td>β-ENaC</td>
<td>IM</td>
<td>100 ± 10^B</td>
<td>143 ± 35^AB</td>
<td>208 ± 26^A</td>
</tr>
<tr>
<td>γ-ENaC (85 kDa)</td>
<td>CTX</td>
<td>100 ± 12^B</td>
<td>185 ± 10^A</td>
<td>194 ± 20^A</td>
</tr>
<tr>
<td>γ-ENaC (85 kDa)</td>
<td>OM</td>
<td>100 ± 10^B</td>
<td>256 ± 10^A</td>
<td>217 ± 17^A</td>
</tr>
<tr>
<td>γ-ENaC (85 kDa)</td>
<td>IM</td>
<td>100 ± 16^B</td>
<td>227 ± 53^AB</td>
<td>313 ± 41^A</td>
</tr>
<tr>
<td>γ-ENaC (70 kDa)</td>
<td>CTX</td>
<td>100 ± 9^B</td>
<td>116 ± 8^B</td>
<td>251 ± 20^A</td>
</tr>
<tr>
<td>γ-ENaC (70 kDa)</td>
<td>OM</td>
<td>100 ± 6</td>
<td>111 ± 8</td>
<td>122 ± 19</td>
</tr>
<tr>
<td>γ-ENaC (70 kDa)</td>
<td>IM</td>
<td>100 ± 16^B</td>
<td>145 ± 35^AB</td>
<td>254 ± 4^A</td>
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</tbody>
</table>

*mean ± sem expressed as percent of control. ^A the mean is significantly (p < 0.05) increased relative to means labeled with “B”. ^B the mean is significantly decreased relative to means labeled with “A”. ^AB the mean is not significantly different from means labeled with either “A” or “B”, as assessed by one-way ANOVA followed by Tukey’s multiple comparisons test.
Figure 2
Figure 3

A. Urine K+Na over days with different treatments.

B. Aldosterone excretion over days with different treatments.

Legend:
- Control
- dDAVP
- dDAVP + WL

Key:
- A
- B

Note:
- Begin dDAVP Infusion
- Begin water-load (only dDAVP + liquid)
Figure 4

![Graph showing mean arterial blood pressure over time with different treatments and markers for statistical significance.](image-url)
Figure 5

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>dDAVP</th>
<th>dDAVP + WL</th>
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</thead>
<tbody>
<tr>
<td>NCC (CTX)</td>
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<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
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<tr>
<td>α-ENaC (CTX)</td>
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<td><img src="image5" alt="Image" /></td>
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<tr>
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</table>
Figure 6

A.

<table>
<thead>
<tr>
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<th>dDAVP</th>
<th>dDAVP + WL</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IM</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B.

Bar chart showing the comparison of protein expression levels (% Control Mean) across different conditions (Control, dDAVP, dDAVP + WL) with error bars indicating variability.
Figure 7