Activation of ENaC by AVP contributes to the urinary concentrating mechanism and dilution of plasma

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Mironova E, Chen Y, Pao AC, Roos KP, Kohan DE, Bugaj V, Stockand JD. Activation of ENaC by AVP contributes to the urinary concentrating mechanism and dilution of plasma. Am J Physiol Renal Physiol 308:F237–F243, 2015. First published November 12, 2014; doi:10.1152/ajprenal.00246.2014.—Arginine vasopressin (AVP) activates the epithelial Na+ channel (ENaC). The physiological significance of this activation is unknown. The present study tested if activation of ENaC contributes to AVP-sensitive urinary concentration. Consumption of a 3% NaCl solution induced hypernatremia and plasma hypertonicity in mice. Plasma AVP concentration and urine osmolality increased in hypernatremic mice in an attempt to compensate for increases in plasma osmolality. ENaC activity was elevated in mice that consumed 3% NaCl solution compared with mice that consumed a diet enriched in Na+. AVP promotes concentration of urine and plasma osmolality through two effects. First, AVP promotes urinary concentration through a feedback response to decreases in arterial blood pressure. AVP controls plasma tonicity by regulating AVP receptor 2 in the distal nephron to concentrate urine by increasing urinary osmolality and decreasing urine flow. AVP increases the water permeability of the collecting duct by increasing the expression of aquaporin (AQP)2 water channels in the luminal membrane of principal cells. AVP induces antidiuresis by stimulating a decrease in both urine water excretion (antiaquaresis) and urine Na+ excretion (antinatriuresis) (5, 17, 27). The physiological significance of AVP controlling urine Na+ excretion is unclear, which raises the following question: if AVP promotes renal Na+ conservation, then how does this process affect plasma tonicity? AVP via AVP receptor 2 decreases Na+ excretion by stimulating the epithelial Na+ channel (ENaC) (4, 5, 7, 15, 19). ENaC, which is expressed along with AQP2 in the luminal membrane of principal cells, serves as the final effector for hormone pathways that fine tune urinary Na+ and water balance.

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pensatory urinary concentration during hyponatremia and contributes to the dilution of plasma during hyponatremia. As such, these findings demonstrate that in addition to serving as a final effector of the RAAS and blood pressure homeostasis, ENaC also plays a key role in water homeostasis by regulating urine concentration and dilution of plasma.

METHODS

Animals. All mouse use and welfare adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (8) following protocols reviewed and approved by the Institutional Animal Care and Use Committees of the University of Texas Health Science Center (San Antonio, TX) and Veterinary Medical Unit of the Veterans Affairs Palo Alto Health Care System (Palo Alto, CA). Adult age- and weight-matched male C57BL/6J mice (~25 g, 6–8 wk old) were used for all experiments. Mice were purchased from Jackson Laboratories. Control mice were maintained with standard chow (0.32% Na⁺, TD.7912, Harlan Teklad) and ad libitum tap water. For some experiments, mice were maintained with a high-Na⁺ diet (2% Na⁺, TD.92034, Harlan Teklad) and ad libitum tap water. Mice were made hyponatremic by consumption of a 3% NaCl drinking solution for 3 days. In some experiments, desmopressin (dDAVP; 1 ng/h) was provided in 0.9% saline for 3–6 days via sterile drinking solution for 3 days. In some experiments, desmopressin (dDAVP; 1 ng/h) was provided in 0.9% saline for 3–6 days via sterile Alzet micro-osmotic pumps (model 1007D, Durect) implanted subcutaneously between the scapulae. In other experiments, mice were water deprived for 24 h. Benzamil (1.4 mg/kg body wt) was provided once a day for 3 days via an intraperitoneal injection in 100 μl water.

Metabolic cage experiments. In vivo analysis of urine and plasma electrolyte and water content followed standard methods previously published (15, 20). In brief, mice (n = 4–5 mice/condition) were housed in metabolic cages (Techniplast) for the duration of the study. Mice were provided 3 days of free access to normal chow and tap water to acclimate before experimentation. During the duration of the experimental period was 3 days. During the acclimation and experimental periods, temperature and lighting (12:12-h light-dark cycle) were controlled. All measurements and procedures were made at a routine time, and mice were provided measured amounts of drinking solution and chow during the experimental period. Spontaneously voided urine was collected under light mineral oil every 24 h. At the end of the experiment, mice were euthanized and trunk blood immediately collected in heparinized tubes via cardiac puncture.

Analysis of hormones and electrolytes. Urinary and plasma Na⁺ concentrations and osmolality were determined using standard procedures with a PFP7 flame photometer (Technec) and vapor pressure osmometer (Wescor). Plasma AVP concentration was quantified with a competitive enzyme-linked immunoassay (Arg8-Vasopressin EIA kit, Enzo Life Sciences) following standard procedures (15). Medullary Na⁺ content was also quantified using standard procedures (1, 24). In brief, kidneys were removed from mice, and the inner medulla was rapidly excised and dissected. Inner medulla samples were blotted on presoaked filter paper, placed in a separate tube, and weighed.

Table 1. Epithelial Na⁺ channel activity

<table>
<thead>
<tr>
<th>Condition</th>
<th>N</th>
<th>Pₒ</th>
<th>Nₒ</th>
<th>n (Total)</th>
<th>f</th>
<th>βNPₒ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.4 ± 0.3*</td>
<td>0.27 ± 0.04</td>
<td>0.73 ± 0.17</td>
<td>65 (141)</td>
<td>0.46*</td>
<td>0.34 ± 0.08</td>
</tr>
<tr>
<td>AVP</td>
<td>3.8 ± 0.3*</td>
<td>0.43 ± 0.02*</td>
<td>1.78 ± 0.22</td>
<td>43 (60)</td>
<td>0.72*</td>
<td>1.28 ± 0.16*</td>
</tr>
<tr>
<td>2% Na⁺ diet</td>
<td>1.5 ± 0.1*</td>
<td>0.16 ± 0.02*</td>
<td>0.31 ± 0.06*</td>
<td>55 (153)</td>
<td>0.36*</td>
<td>0.11 ± 0.02*</td>
</tr>
<tr>
<td>3% NaCl water</td>
<td>2.0 ± 0.3+</td>
<td>0.15 ± 0.02</td>
<td>0.45 ± 0.13</td>
<td>26 (49)</td>
<td>0.53†</td>
<td>0.24 ± 0.06†</td>
</tr>
</tbody>
</table>

The control condition represents tubules harvested from control mice provided 0.32% Na⁺ diet and ad libitum water; the arginine vasopressin (AVP) condition represents tubules harvested from control mice treated with 1 μM AVP for 30 min; the 2% Na⁺ diet condition represents tubules harvested from mice provided a 2% Na⁺ diet and ad libitum tap water for 5 days; and 3% NaCl water condition represents tubules harvested from mice provided 3% NaCl drinking solution and 0.32% Na⁺ diet for 3–4 days. n is the number of patches with at least one active epithelial Na⁺ channel; total is the total number of viable seals for that condition. f = n/total, fNPₒ is cellular activity, which is activity (NPₒ) multiplied by the frequency (f) of forming a viable seal for the condition that contained at least one active channel. *Significantly different compared with control; †significantly different compared with 2% Na⁺ diet.
ENaC contributes to dilution of plasma

A

Fig. 2. Treatment with 3% NaCl drinking solution causes hypernatremia and increases plasma AVP concentration. A–D: summary graphs of plasma osmolality (POsm, A), plasma Na⁺ concentration (PNa; B), urinary Na⁺ concentration (UNa; C), and plasma AVP concentration (D) for mice provided tap water (control) or a drinking solution containing 3% NaCl for 3 days. *Significant increase vs. control.

B

RESULTS

AVP activates ENaC. We and others have previously reported that AVP activates ENaC (18, 19, 27). The results shown in Fig. 1 are in agreement with these previous findings. Shown in Fig. 1A is a continuous current trace before and after the addition of AVP to the bath solution of a cell-attached patch that contained at least five ENaC. This patch was formed on the apical membrane of a principal cell in an isolated, split-open murine distal nephron. In this representative experiment and in the summary results shown in Fig. 1B and Table 1, AVP increases ENaC activity by significantly increasing both channel number and open probability.

AVP maintains ENaC activity high during hypernatremia. In a recent study (15) of adrenalectomized mice, we demonstrated that ENaC was active in the absence of aldosterone due to a rise in plasma AVP concentration. Adrenalectomy, however, introduces a multitude of changes, including a loss of all adrenal hormones and a marked decline in plasma volume. In turn, these changes may induce a complex set of adaptations, all of which may obscure a clear understanding of the physiological consequences of AVP regulation of ENaC. To define the physiological consequences of AVP stimulation of ENaC, we fixed Na⁺ and water intake together by maintaining mice with a 3% NaCl drinking solution. This maneuver simultaneously suppresses RAAS signaling while stimulating AVP release because mice become volume expanded and hypernatremic (see below). As a consequence, this experimental perturbation isolates AVP regulation of ENaC and limits confounding influences from the RAAS and other signaling pathways. As shown by the summary graphs in Fig. 2, when mice are forced to consume 3% NaCl solution, plasma tonicity, plasma and urinary Na⁺ concentrations, and plasma AVP concentration

C

**Fig. 3. ENaC activity is high in hypernatremic mice.** Shown is a summary graph of total cellular ENaC activity in mice maintained with normal chow and ad libitum tap water (control), 2% dietary Na⁺ and ad libitum tap water, and normal chow and 3% NaCl drinking solution for 3 days. Patch-clamp conditions were identical to those described in Fig. 1. *Significantly less than control; **significantly greater than 2% dietary Na⁺.
increase. While the RAAS is suppressed under these conditions, the rise in AVP concentration is consistent with preservation of some ENaC activity in this state of hypernatremia and with a role for AVP-stimulated ENaC in counteracting plasma hypertonicity by facilitating renal water reabsorption, urine concentration, and dilution of plasma.

The results shown in Fig. 3 and Table 1 reveal that ENaC is indeed active in hypernatremic mice maintained with a 3% NaCl drinking solution. Figure 3 and Table 1 show ENaC activity in distal nephron principal cells from mice maintained with normal chow and ad libitum tap water (control), ad libitum tap water and a high-Na⁺ (2% Na⁺) diet, and 3% NaCl solution and normal chow. Consistent with previous findings, ENaC activity is suppressed when mice are allowed to consume water as they increase Na⁺ intake (6, 16, 30). When mice consume a Na⁺-rich diet, plasma volume expands and RAAS signaling is suppressed, which leads to an increase in urine Na⁺ excretion associated with a decrease in ENaC activity. With ad libitum water consumption, mice are able to regulate water balance independently: plasma Na⁺ concentration and plasma AVP concentration do not change. However, when mice are maintained with a 3% NaCl drinking solution, Na⁺ and water intake are fixed so that both volume expansion and hypernatremia ensue. In this condition, an increase in serum AVP concentration is able to preserve some ENaC activity despite suppression of the RAAS. Consumption of a Na⁺-rich diet leads to volume expansion and suppression of RAAS signaling, whereas the concomitant hypernatremia increases plasma AVP concentration, which stimulates ENaC activity.

Activation of ENaC by AVP facilitates concentration of urine. As shown in Fig. 4, active ENaC contributes to compensatory water conservation during hypernatremia. Figure 4 shows urine osmolality and flow in mice maintained with ad libitum water and normal chow compared with those maintained with 3% NaCl drinking solution in the absence and presence of benzamil treatment. Consumption of hypertonic saline promotes urine concentration as reflected by an increase in urine osmolality. Treatment with benzamil significantly decreases urine osmolality and increases urine flow in mice with 3% NaCl drinking solution.

ENaC-dependent urinary concentration contributes to dilution of plasma. As shown above, forced consumption of hypertonic saline causes hypernatremia; benzamil impedes urinary concentration in this condition. From these observations, we predicted that treatment of mice that consumed 3% NaCl drinking solution with benzamil should increase plasma Na⁺ concentration further. As demonstrated by the results shown in Fig. 5, this was the case. Figure 5A shows plasma Na⁺ concentration from mice maintained with ad libitum water and normal chow compared with those maintained with 3% NaCl solution and 3% NaCl solution plus benzamil treatment. Consumption of 3% NaCl solution increases plasma Na⁺ concentration; treatment of these mice with benzamil significantly increases plasma Na⁺ concentration further. Similarly, as shown in Fig. 5B, administration of benzamil to mice that consumed 3% NaCl solution significantly increases further the already elevated plasma osmolality.

Mechanism. Inhibition of ENaC diminishes the draw for reabsorption of urinary water. The results shown in Fig. 6 reveal the mechanism by which AVP-stimulated ENaC contributes to urinary concentration. Consumption of hypertonic saline increases medullary Na⁺ content and urinary Na⁺ ex-
cretion. Benzamil further significantly increases renal Na\(^+\) excretion in mice maintained with a 3% NaCl drinking solution without affecting medullary Na\(^+\) content. Thus, the mechanism by which benzamil impairs urine concentration does not involve impairment of the medullary Na\(^+\) concentration gradient; rather, inhibition of ENaC impairs tubular transport of Na\(^+\) so that Na\(^+\) is trapped in the urine, lessening the osmotic gradient between urine and the interstitial fluid. Because this Na\(^+\) in urine is osmotically active, inhibition of ENaC results in retaining water in the urine without changing urinary Na\(^+\) concentration. Such observations demonstrate that by inhibiting ENaC, benzamil promotes an osmotic diuresis and consequently decreases urine concentrating ability, making the Na\(^+\) concentration in the plasma more like the fluid being consumed. When this fluid is hypertonic saline, inhibition of ENaC further increases plasma Na\(^+\) concentration and worsens hypernatremia by limiting concentrating ability.

Facilitation of urinary concentrating ability is a general manifestation of AVP stimulation of ENaC. The findings above are consistent with a role for AVP-stimulated ENaC in facilitating reabsorption of urinary water to compensate for hypernatremia. To test whether facilitation of urinary concentrating ability is a general manifestation of AVP stimulation of ENaC and to test whether AVP-stimulated ENaC plays a role in the pathogenesis of hyponatremia, we water deprived mice and injected mice with dDAVP in the presence and absence of benzamil. As shown in Fig. 7, urine osmolality increases and urine flow decreases in both water-deprived and dDAVP-treated mice, demonstrating the expected concentration of urine in response to these stimuli. During water deprivation, urinary concentration is an appropriate compensatory response, whereas in dDAVP-treated mice, urinary concentration is causative for hyponatremia (11). Treatment with benzamil significantly reduces urine osmolality and increases urine flow in

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**Fig. 6.** Inhibition of ENaC decreases the draw for water reabsorption in hypernatremic mice. **A–C:** summary graphs of medullary Na\(^+\) content (**A**), urinary Na\(^+\) excretion (**B**), and urinary Na\(^+\) concentration (**C**) in matched mice maintained with tap water (control), 3% NaCl drinking solution, or 3% NaCl solution plus injection with benzamil for 3 days. *Significantly greater compared with control; **significantly greater compared with the absence of benzamil.

**Fig. 7.** Inhibition of ENaC blunts AVP-dependent urinary concentration. **A and B:** summary graphs of urine osmolality (**top**) and urine flow (**bottom**) in untreated control mice and matched mice water deprived for 24 h (**A**) or instrumented with a minipump releasing desmopressin (dDAVP) for 3 days (**B**) in the absence and presence of injection with benzamil. *Significantly different from control; **significantly different compared with the absence of benzamil.
water-deprived mice; treatment with benzamil significantly reduces urine osmolality and promotes a trend toward increased urine flow in dDAVP-treated mice. Thus, under both conditions, inhibition of ENaC compromises urinary concentrating ability. These results are consistent with a role for AVP-mediated activation of ENaC in not only promoting renal water retention under conditions of hypernatremia but also for causing hyponatremia by obligating renal water reabsorption and urinary concentration. Thus, AVP activation of ENaC is a general response for promoting renal water conservation and dilution of plasma.

**DISCUSSION**

The following questions inspired the present study: 1) what is the function of ENaC when AVP signaling is active; 2) does AVP-mediated activation of ENaC serve to increase renal water reabsorption during hypernatremia and AVP-driven hyponatremia; or 3) does inhibition of ENaC relieve hypernatremia and exacerbate hyponatremia? The answers to these questions are not obvious because ENaC is primarily regarded as an end effector of RAAS signaling. Moreover, inhibition of ENaC could relieve hypernatremia and exacerbate hyponatremia by decreasing Na⁺ content in the plasma. However, the present results demonstrate that this was not the case. Inhibition of ENaC exacerbates hypernatremia and relieves hyponatremia because the primary action of AVP-mediated ENaC activation is to facilitate urine concentration and dilution of plasma.

The present findings are consistent with the recent observation that AVP maintains ENaC activity in adrenalectomized mice (15). Such animals are hypovolemic and hyponatremic because mineralocorticoids are absent and AVP assumes a greater role in stimulating both renal Na⁺ and water retention. However, due to the complex physiological adaptations that occur with adrenal insufficiency, it remains unclear from this earlier study whether stimulation of ENaC by AVP exacerbated hyponatremia while compensating for volume depletion or if activation of ENaC mitigated both decreases in plasma volume and tonicity. This uncertainty highlights the inherent limitation of how all biological organisms, including terrestrial vertebrates, must use solutes such as Na⁺ to absorb water and regulate water homeostasis. When water can move only through osmosis, the need to protect plasma volume may compete with the need to protect plasma tonicity. ENaC sits at the nexus of these two homeostatic systems, one controlling plasma tonicity and the other plasma volume. A central question that emerges from this paradigm: what is the activity of ENaC and the consequences of this activity when the need to maintain volume competes against the need to maintain tonicity?

The present findings show that AVP-mediated activation of ENaC facilitates urinary concentration and dilution of plasma. In instances where AVP drives hypernatremia, ENaC is positioned to maintain or even exacerbate hypernatremia by promoting urine concentration. This may seem counterintuitive if one only considers the role of ENaC in RAAS signaling and plasma volume regulation. However, if one considers a broader role of ENaC in mediating AVP action on water homeostasis, as explored in the present study, it becomes apparent that inhibitors of ENaC provoke osmotic diuresis because inhibition of ENaC compromises urinary concentrating ability to some degree. In this sense, inhibitors of ENaC are similar to all diuretics in that they compromise the ability of the kidney to regulate Na⁺ concentration in urine and consequently force Na⁺ concentration of plasma to be more similar to that in the fluid being ingested.

The present findings may have important implications for the treatment of hyponatremia associated with high AVP levels such as in decompensated heart failure, cirrhosis, and syndrome of inappropriate antidiuretic hormone secretion. In these diseases, where hyponatremia is the clinical hallmark of the nonosmotic release of AVP, inhibition of ENaC may serve to counter hyponatremia at the same time as decreasing plasma volume. This strategy may be particularly useful in heart failure and cirrhosis, where aldosterone levels are concomitantly elevated (25, 28, 29, 31). Mineralocorticoid receptor antagonists are frequently used to inhibit aldosterone action in these diseases, but these treatments do not necessarily block the action of AVP, which is also increased. The increase in AVP levels in these diseases may act in a synergistic manner with aldosterone to increase pathologic renal Na⁺ and water retention. A combined regimen of antagonists against ENaC and mineralocorticoid receptors could remove the risk of hyponatremia by increasing both urine NaCl and water excretion.

We propose that ENaC contributes to dilution of plasma when the channel, along with AQP2, is activated by AVP. This notion is consistent with what was reported many years ago when both aldosterone and AVP were considered to be required for maximal urine concentration (for a review, see Ref. 26). As we and others have shown, ENaC activity is maximal in the presence of both aldosterone and AVP (7, 10, 19, 22, 23). We argue that this regulation contributes to the underlying basis for the standing clinical maxim that the kidney protects volume over tonicity. AVP-mediated activation of ENaC serves to increase renal Na⁺ retention and protect plasma volume, but this can occur at the expense of increasing renal water retention. This mechanism may contribute to hyponatremia observed in individuals with significant volume depletion. In addition, we propose that AVP-mediated activation of ENaC also serves to dilute plasma by facilitating maximal urinary concentration. This mechanism may exacerbate hyponatremia in diseases with high AVP levels, such as in heart failure, cirrhosis, or syndrome of inappropriate antidiuretic hormone secretion.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


