Escape from vasopressin-induced antidiuresis: role of vasopressin resistance of the collecting duct

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Ecelbarger, Carolyn A., Chung-Lin Chou, Alanna J. Lee, Susan R. DiGiovanni, Joseph G. Verbalis, and Mark A. Knepper. Escape from vasopressin-induced antidiuresis: role of vasopressin resistance of the collecting duct. Am. J. Physiol. 274 (Renal Physiol. 43): F1161–F1166, 1998.—Previously, we demonstrated that escape from vasopressin-induced antidiuresis (“vasopressin escape”) in rats is associated with a large, selective decrease in whole kidney expression of aquaporin-2, the vasopressin-regulated water channel. Here, we show that isolated perfused inner medullary collecting ducts (IMCDs) from vasopressin-escape rats (desamino-[d-arginine]vasopressin (DDAVP)/water-loaded) have dramatically reduced vasopressin-dependent osmotic water permeabilities [46% of control rats (DDAVP alone)], which coincides with a fall in inner medullary aquaporin-2 protein abundance as measured by immunoblotting in the opposite kidney. Furthermore, we demonstrate in IMCD suspensions that cAMP accumulation in response to DDAVP is substantially reduced in the vasopressin-escape rats both in the presence and absence of the phosphodiesterase inhibitor IBMX. By immunoblotting, we show that the abundance of two proteins important in cAMP generation: the stimulatory heterotrimeric G protein subunit Gαs and adenylyl cyclase type VI, do not change. We conclude that vasopressin escape is associated with relative vasopressin resistance of the collecting duct cells manifested by decreased intracellular cAMP levels. The decreased cAMP levels contribute to the demonstrated decrease in collecting duct water permeability in two ways: 1) by causing a decrease in aquaporin-2 expression and 2) by limiting the acute action of vasopressin to increase collecting duct water permeability.

IN_patients_and_experimental_animals, inappropriately high levels of circulating vasopressin with sustained water intake can result in net water retention and the development of hyponatremia. However, the degree of water retention is limited by a physiological phenomenon, “vasopressin escape,” through which the kidney decreases water excretion and decreases urinary osmolality, despite sustained high circulating levels of vasopressin. Our recent studies have demonstrated that the vasopressin-escape phenomenon is a consequence of decreased expression of aquaporin-2 (8).

Aquaporin-2 is the principal water channel of the apical plasma membrane of kidney collecting duct principal cells. Vasopressin acutely increases the permeability of the collecting duct to water by stimulating exocytic insertion of aquaporin-2 into the apical plasma membrane, allowing increased water absorption from the collecting duct lumen into the blood (13). Vasopressin also has a long-term action to increase the number of aquaporin-2 water channels per collecting duct principal cell (7, 14). It is known that the aquaporin-2 gene contains a CRE (i.e., a cAMP regulatory element) in its 5′-flanking region, raising the possibility that the expression of this protein may be regulated by intracellular cAMP levels (10, 12).

During the development of the vasopressin-escape phenomenon, aquaporin-2 water channel protein and mRNA abundance in the renal collecting duct are dramatically reduced, coincident with the observed increase in water excretion (8). Based on these observations, we concluded that the reduction in expression of aquaporin-2 protein is an important mediator of vasopressin escape. We speculated that the fall in aquaporin-2 protein may be accomplished through control of aquaporin-2 gene transcription rate, possibly via a lowering of intracellular cAMP levels.

The purpose of these studies was to test the hypothesis that the vasopressin-escape process is associated with relative vasopressin resistance of the collecting duct cells, involving a decrease in vasopressin-stimulated cAMP production and a decrease in vasopressin-dependent water permeability. We address this question using 1) the isolated perfused tubule technique to measure vasopressin-dependent water permeability in inner medullary collecting ducts (IMCDs); 2) measurements of the cAMP response to desamino-[d-arginine]vasopressin (DDAVP) in IMCDs; and 3) immunoblotting to assess the expression levels of aquaporin-2 and of two regulatory proteins, the heterotrimeric G protein subunit Gαs and adenylyl cyclase type VI. The results demonstrate a marked vasopressin resistance of IMCDs from vasopressin-escape rats and confirm that the vasopressin-escape phenomenon is associated with a decrease in aquaporin-2 expression.

MATERIALS AND METHODS

Experimental animals. All experiments were conducted in accord with an animal protocol (5-KE-1) approved by the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute. Under light methoxyflurane anesthesia, male Sprague-Dawley rats (Taconic Farms, Germantown, NY) were subcutaneously implanted with osmotic minipumps (model 2002; Alzet, Palo Alto, CA), which delivered 20 ng DDAVP (Peninsula Laboratories, Belmont, CA) per hour. Four days later, rats were split into two treatment
groups: “escape” and “control.” Vasopressin-escape rats were given a daily water load via a gelled-agar diet (71% water, 28% finely-ground rat chow, 1% agar, BACTO-AGAR; Difco Laboratories, Detroit, MI), based on an approach originally designed by Bouby et al. (3). They received 52–65 g of this concoction per 250 g body wt per day. The diet forced the rats to consume a greater volume of water to take in the calories which they desired. Control rats were given same amount of the dry components of the diet, i.e., agar and chow. They received water only on an ad libitum basis. Urine was collected in the final 24 h for measurement of osmolality and volume. After 4 days of treatment, the rats were killed.

Antibodies. Polyclonal aquaporin-2 antibody (L127) was raised in rabbit against a synthetic peptide corresponding to the terminal 22 amino acids of aquaporin-2 protein. Conditions for use and characterization of this antibody have been previously reported (7). A polyclonal antibody against Gα protein (16) was kindly provided by Dr. Paul Goldsmith at the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD. A polyclonal adenylyl cyclase V/VI antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Electrophoresis and immunoblotting of proteins. The rats were killed by decapitation, and trunk blood was collected for serum. Both kidneys were rapidly removed and either processed immediately or frozen in dry ice for later processing. The inner medulla was dissected from the right kidney with sharp, curved scissors. Homogenates for immunoblotting were prepared from the whole left kidneys and from the inner medulla of the right kidney as previously described (8). Homogenized tissue was diluted to a protein concentration of 1 µg/µl with sucrose/ triethanolamine buffer, pH 7.6, mixed with 5 X Laemmli sample buffer (4 vol sample to 1 vol Laemmli buffer), and heated to 60°C for 15 min to solubilize proteins. SDS-PAGE was done with Bio-Rad prestac 7.5 and 12% gels (Bio-Rad Laboratories, Hercules, CA). Conditions for immunoblotting and chemiluminescent protein detection have been previously described (9). Detection reagents were provided as kits: LumiGlo (Kirkegaard and Perry Laboratories, Gaithersburg, MD) or SuperSignal (Pierce, Rockford, IL).

Perfusion of microdissected IMCD segments. Collecting ducts were dissected from the mid-portions of the inner medulla and were perfused at 37°C by the method of Burg et al. (4). The osmotic water permeability (Pf) was measured using 1 mM fluorescein sulfonate as a luminal volume marker as described by Wall et al. (17). The peritubular bath was identical to the perfusate, except that it did not contain fluorescein sulfonate and had sufficient additional NaCl to increase its osmolality to 490 mosmol/kgH2O, i.e., 200 mosmol/kgH2O greater than the perfusate. The fluorescein sulfonate concentration was measured in perfusate and collected fluid using a continuous flow fluorometer (coefficient of variation, 2%). Pf was calculated using the equation of Al-Zahid et al. (1). The peritubular bath contained 100 µM arginine vasopressin (AVP, Peninsula). After 30-min equilibration at 37°C in the presence of AVP, three collections were made to determine the AVP-dependent Pf.

cAMP measurement in IMCD suspensions. Intracellular cAMP levels were measured in IMCD suspensions prepared from both groups of rats as previously described (6). An individual suspension was prepared for each animal, and 50-µl aliquots of each rat’s suspension were incubated under eight conditions: 0, 0.1, 1.0, and 10 nM DDAVP in the presence or absence of 0.25 mM IBMX, a phosphodiesterase inhibitor. After 5 min at 37°C, the incubation was terminated by adding 200 µl of 10% trichloroacetic acid and placing the sample tube on ice. cAMP levels in the supernatant were determined by a radioimmunoassay (Amersham Life Sciences, Arlington Heights, IL). Protocol details have been previously described (6). Protein content (Bradford method, Bio-Rad 500-006) of each suspension aliquot was measured for normalization.

Statistics. Immunoblot band densities were measured by laser densitometry (Molecular Dynamics, San Jose, CA). Statistical significance was assessed by an unpaired t-test or Welch’s t-test when standard deviations of the two groups were different. cAMP data was analyzed by two-way analysis of variance utilizing the two factors: 1) treatment (escape or control) and 2) DDAVP dose. For all cases, P < 0.05 was considered significant.

RESULTS

Table 1 summarizes physiological measurements for vasopressin-escape (DDAVP-treated/water-loaded) rats versus control rats (treated with DDAVP alone). Urine volume was significantly increased and urine osmolality significantly decreased after 4 days of water loading in the presence of DDAVP. Likewise, similar to earlier studies, serum osmolality for vasopressin-escape rats was extremely low in response to the water loading.

We assessed aquaporin-2 protein abundance in whole kidney homogenates from the rats described in Table 1 by immunoblotting (Fig. 1A). In agreement with our previous studies (8), after 4 days of water loading, the escape rats had dramatically decreased whole kidney aquaporin-2 expression. The summed band density (29-kDa band plus the 35- to 45-kDa glycosylated band) for the vasopressin-escape rats was only 28 ± 3% of that for the controls. An immunoblot of homogenates from the inner medullas prepared from the right kidney of these rats is shown in Fig. 1B. The summed band density of values from vasopressin-escape rats as a percent of the control mean was 72 ± 7%. Thus, although aquaporin-2 expression was significantly reduced in the inner medullas of the vasopressin-escape rats relative to their controls, the decrease was less pronounced than that in the kidney as a whole.

To test whether the decrease in aquaporin-2 expression corresponds to a fall in water permeability of the collecting ducts, we measured the osmotic water permeability of IMCDs from six vasopressin-escape rats and six controls using the isolated perfused tubule technique. We found that, with 100 nM vasopressin in the peritubular bath, the osmotic water permeabilities of the IMCDs from the vasopressin-escape rats were reduced on average to 46% of their corresponding controls (vasopressin-escape, 240 ± 25 µm/s; control, 527 ± 63 µm/s; P < 0.002, Fig. 2). Immunoblots for

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body Weight, g</th>
<th>Serum Osmolality, mosmol/kgH2O</th>
<th>Urine Osmolality, mosmol/kgH2O</th>
<th>Urine Volume, ml/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>242 ± 16</td>
<td>285 ± 3</td>
<td>2,926 ± 187</td>
<td>5.2 ± 0.4</td>
</tr>
<tr>
<td>Escape</td>
<td>238 ± 17</td>
<td>233 ± 4</td>
<td>1,812 ± 202</td>
<td>9.3 ± 1.4</td>
</tr>
<tr>
<td>P Value</td>
<td>0.86</td>
<td>&lt;0.0001*</td>
<td>0.037*</td>
<td>0.026*</td>
</tr>
</tbody>
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Values are means ± SE; n = 5 rats per treatment. *Statistically significant difference between the two treatments.
aquaporin-2 in the inner medullas of the right kidneys from the same rats revealed that the aquaporin-2 band density for vasopressin-escape rats was 47 ± 6% of controls (immunoblot not shown). This evidence strongly supports the conclusion that reduced aquaporin-2 levels in the collecting ducts of vasopressin-escape rats are associated with decreased epithelial water permeabilities and provides an explanation for the decrease in water retention in vasopressin-escape rats.

Next, we measured cAMP production in IMCD suspensions prepared from vasopressin-escape rats (chronic DDAVP plus water load) and their respective controls (DDAVP only). Fifty-microliter aliquots of suspensions were incubated for 5 min with four different doses of DDAVP (0, 0.1, 1, and 10 nM) in the presence (Fig. 3A) or absence (Fig. 3B) of 0.25 mM IBMX, a phosphodiesterase inhibitor. Whether in the presence or absence of IBMX, there was a significant reduction in the cAMP accumulation in tubules from vasopressin-escape rats. Because the difference between the treatments was no less apparent in the presence of the phosphodiesterase inhibitor, IBMX, we conclude that the collecting ducts from the vasopressin-escape rats have a decreased ability to produce cAMP, i.e., a relative vasopressin resistance.

A reduction in cAMP production in the IMCD could have several possible causes including decreased expression of any of the proteins important in cAMP generation. Therefore, we assessed by immunoblotting the relative abundance of the GTP-binding protein (G₅₆) and of adenyl cyclase VI in inner medullary homogenates from vasopressin-escape versus control rats (Fig. 4). [Chabardes et al. (5) have previously demonstrated that the predominant adenyl cyclase isotype expressed in the collecting duct is type VI.] There was no significant difference between the groups for either of these two proteins. Densities of corresponding bands for vasopressin-escape rats expressed as a percentage of their controls were as follows: G₅₆, 137 ± 6%, P = 0.12; and adenyl cyclase, 106 ± 22%, P = 0.81. Therefore, regulation of the abundance of either of these two proteins is unlikely to play a role in decreased cAMP production by the IMCD suspensions.

**DISCUSSION**

Escape from vasopressin-induced antidiuresis is a critical means by which the body is able to limit water retention when moderate or high levels of water are consumed in the continual presence of high circulating levels of blood vasopressin. Inappropriate levels of vasopressin (from the point of view of osmotic regulation) are seen in a large number of clinical conditions including the syndrome of inappropriate antidiuretic hormone secretion (SIADH), congestive heart failure, cirrhosis, and nephrotic syndrome (2). Physiological...
parameters characteristic of vasopressin-escape include increased urine volume and decreased urine osmolality, both of which were documented in the present animal study (see Table 1).

Until recently, little was known about mechanisms involved in the vasopressin-escape phenomenon. Recently, we demonstrated in a rat model that vasopressin escape was correlated temporally with a dramatic fall in aquaporin-2 protein and mRNA levels in the kidney (8). Based on the fall in aquaporin-2 expression, we proposed that collecting duct water permeability was decreased, thus impairing free water absorption in the collecting ducts of vasopressin-escape rats. In the present studies, we confirmed that aquaporin-2 expression is decreased in association with the vasopressin-escape phenomenon (Fig. 1) and demonstrated directly that the decrease in aquaporin-2 expression is associated with a marked decrease in maximal osmotic water permeability of the IMCDs (Fig. 2). The fall in maximal water permeability is likely due to the decrease in aquaporin-2 expression, although the lower level of cAMP in the cells may also limit the trafficking of aquaporin-2 to the apical plasma membrane.

IMCD from vasopressin-escape rats exhibited decreased cAMP production. cAMP accumulation in the presence of the phosphodiesterase inhibitor, IBMX (0.25 mM). A decrease was also seen in the absence of IBMX (Fig. 3B). Thus IMCDs exhibited partial resistance to vasopressin. This conclusion was further supported by the finding of a decreased vasopressin-dependent water permeability in isolated perfused collecting ducts from vasopressin-escape rats (Fig. 2). The apparent vasopressin resistance of the collecting duct cells resulting in decreased cellular cAMP could play a role in the decrease in expression of aquaporin-2

Fig. 3. cAMP accumulation in IMCD suspensions of vasopressin-escape rats (DDAVP/water loaded; □) and their controls (DDAVP alone; ■) after incubation with DDAVP or vehicle. A: 5-min incubation in the presence of the phosphodiesterase inhibitor, IBMX (0.25 mM). B: 5-min incubation in the absence of IBMX. In both cases, 50-µl aliquots of IMCD suspension were challenged with one of four doses of DDAVP (0, 0.1, 1, and 10 nM). The reaction was terminated by the addition of trichloroacetic acid. cAMP accumulation was significantly reduced in the vasopressin-escape rats throughout the dose profile, both in the presence and absence of IBMX.
mRNA and protein via a reduction in cAMP-stimulated transcription rate. cAMP activates protein kinase A, which results in the phosphorylation of CREB (i.e., cAMP regulatory binding protein), and possibly other transcription factors (10, 12, 18). The phosphorylated transcription factors are hypothesized to bind to the 5'-flanking region of the aquaporin-2 gene and to increase the rate of aquaporin-2 transcription.

A reduction in cellular cAMP accumulation could theoretically result from either increased degradation of cAMP via enhanced cyclic nucleotide phosphodiesterase activity or from decreased adenylyl cyclase activity. The suspensions prepared from the vasopressin-escape rats had significantly depressed ability to accumulate cAMP regardless of whether cyclic nucleotide phosphodiesterases were inhibited. Thus it appears that an increase in endogenous phosphodiesterase activity cannot be the main factor responsible for the apparent vasopressin resistance of the cells and the fall in intracellular cAMP. Rather, we conclude the vasopressin-escape is associated with decreased cAMP production via a decrease in adenylyl cyclase activity.

The mechanism by which vasopressin-dependent cAMP production is decreased in collecting ducts of vasopressin escape rats could not be determined from the present study. Theoretically, the decrease could involve changes in several regulatory factors including the heterotrimeric G proteins (Gα and Gβγ), intracellular Ca2+ concentration [which can directly inhibit some adenylyl cyclases including the collecting duct form, adenylyl cyclase type VI (5)], the vasopressin receptor, or adenylyl cyclase itself. Our immunoblotting studies did not reveal changes in Gα or adenylyl cyclase VI abundance, although our results do not rule out involvement of these proteins, since their activities could be altered by mechanisms other than regulation of their abundance. It appears likely that altered regulation of the vasopressin receptor (V2 isotype) could be involved in the escape phenomenon, although this hypothesis has not been tested in the present study. The immunoblotting experiments demonstrated a greater escape-associated suppression of aquaporin-2 in homogenates of whole kidney than in homogenates of inner medulla. The greater suppression of aquaporin-2 expression in whole kidney versus inner medulla suggests that the escape-associated suppression of water permeability may be greatest in the proximal portion of the collecting duct system, which would be represented in the whole kidney homogenates but not in the inner medullary homogenates. This result suggests that the suppression of water permeability may be even greater in the cortical than in the medullary collecting duct. Based on the prior micropuncture findings of J. Amison et al. (11) in Brattleboro rats, such an effect would be expected to shift collecting duct water absorption from the cortex to medulla, resulting in increased water absorption in the IMCD (despite a decrease in water permeability) as a result of enhanced transepithelial osmotic gradients. The increase in IMCD water absorption would be expected to result in dilution of medullary interstitial solutes (see Ref. 15), thereby contributing to the loss of concentrating ability. Further studies will be required to test this model.

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