Deceased vasopressin-mediated renal water reabsorption in rats with chronic aldosterone-receptor blockade

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Increased plasma aldosterone level is considered among the most important mechanisms involved in the decreasing ability to generate a maximally concentrated urine as shown in patients with Addison’s disease (49) or in adrenalectomized animals (42). Treatment with aldosterone-receptor antagonists like spironolactone increases dilute urine production (48). However, the mechanism behind the impaired renal concentrating ability during adrenal insufficiency or aldosterone-receptor blockade is unknown.

Studies in isolated collecting ducts (CD) suggest that the presence of mineralocorticoids is needed for a normal action of vasopressin on transepithelial osmotic water permeability (5, 37, 42). However, the mechanism behind this action of mineralocorticoids on CD water permeability is unknown. Furthermore, in vivo micropuncture studies on isolated segments of the thick ascending limb of Henle’s (TAL) and medullary collecting ducts (CD) have demonstrated that aldosterone stimulates sodium transport in rat medullary TAL (44, 51). This suggests that aldosterone is involved in the regulation of the interstitial hyperosmolality in the renal medulla, which is the driving force for the transepithelial water transport across the CD epithelium. Therefore, the lack of stimulation with mineralocorticoids on both TAL and CD segments may be involved in the impaired concentrating ability during adrenal insufficiency.

Recently, a number of studies have shown that vasopressin stimulates water reabsorption in the CD principal cells by vasopressin type 2 (V2) receptor-mediated stimulation of aquaporin-2 (AQP2) water channels (30). AQP2 is localized in the apical plasma membrane and in cytoplasmic vesicles, and acute increases in the plasma vasopressin concentration are associated with insertion of AQP2 from cytoplasmic vesicles into the apical plasma membrane (30). AQP2 is localized in the apical plasma membrane and in cytoplasmic vesicles, and acute increases in the plasma vasopressin concentration are associated with insertion of AQP2 from cytoplasmic vesicles into the apical plasma membrane (30). AQP2 is localized in the apical plasma membrane and in cytoplasmic vesicles, and acute increases in the plasma vasopressin concentration are associated with insertion of AQP2 from cytoplasmic vesicles into the apical plasma membrane (30). AQP2 is localized in the apical plasma membrane and in cytoplasmic vesicles, and acute increases in the plasma vasopressin concentration are associated with insertion of AQP2 from cytoplasmic vesicles into the apical plasma membrane (30). AQP2 is localized in the apical plasma membrane and in cytoplasmic vesicles, and acute increases in the plasma vasopressin concentration are associated with insertion of AQP2 from cytoplasmic vesicles into the apical plasma membrane (30).
avid sodium and water retention in patients with severe congestive heart failure or decompensated liver cirrhosis. This provides the rationale for the use of aldosterone-receptor antagonists in the management of edema, ascites, and hyponatremia in these clinically important conditions (2, 13, 47). However, our understanding of the mechanism by which aldosterone-receptor antagonists affect renal water handling in such conditions is still incomplete.

In the present study, V_2-receptor-mediated water reabsorption in the CD was examined in chronically instrumented rats treated by continuous intravenous infusion (20 mg/day) for 4 wk with the aldosterone-receptor antagonist canrenoate. Experiments were performed in normal Wistar rats and in rats with liver cirrhosis induced by common bile duct ligation (CBL). Untreated rats were used as controls. Acute V_2-receptor blockade was induced by intravenous administration of the selective V_2-receptor antagonist OPC-31260. V_2-receptor blockade was achieved in the absence of changes in fluid balance, by use of a computer-driven, servo-controlled intravenous volume replacement system that replaced urinary losses momentarily by intravenous infusion of 150 mM glucose. In an additional group of animals, the expression of the vasopressin-sensitive water channel AQP2 was determined by semi-quantitative immunoblotting.

METHODS

Materials. Barrier-bred and specific pathogen-free female Wistar rats (210–230 g) were obtained from the Department of Experimental Medicine, Panum Institute, University of Copenhagen (Copenhagen, Denmark). The animals were housed in a temperature (22–24°C) - and moisture (40–70%)-controlled room with a 12:12-h light-dark cycle (light on from 6:00 AM to 6:00 PM). All animals were given free access to tap water and pelleted rat diet containing 140 mmol/kg sodium, 275 mmol/kg potassium, and 23% protein (Altromin catalog no. 1310; Altromin International, Lage, Germany).

Animal preparation. During halothane-nitrous oxide anesthesia, a Silastic catheter was implanted in the left external jugular vein in the rats subjected to chronic aldosterone-receptor blockade. The venous catheter was connected to an osmotic minipump (Alzet model 2ML4; pumping rate 2.5 µl/h; Alza, Palo Alto, CA) that was filled with potassium canrenoate (Searle Scandinavia, Malmö, Sweden) at a concentration that produced an infusion rate of 20 mg base/24 h. Three weeks after CBL or sham-CBL, all rats were anesthetized with halothane-nitrous oxide, and permanent medical-grade Tygon catheters were implanted in the abdominal aorta and in the urinary bladder and was sealed with a silicone-coated stainless steel pin after the bladder was flushed with 0.6 mg/ml ampicillin (Anhypen; Nycomed Pharma, Oslo, Norway). Catheters were produced, fixed, and sealed as described previously (36). After instrumentation, the animals were housed individually. All surgical procedures were performed during aseptic conditions. To relieve postoperative pain, rats were treated with 0.2 mg/kg body wt ip buprenorfine (Anorfin; GEA, Copenhagen, Denmark), and, to accelerate postoperative recovery, animals were given access to 1.5% sodium chloride in addition to tap water until they reached preoperative weight (3–4 days later).

Efficacy of aldosterone-receptor blockade. To investigate the degree of aldosterone-receptor blockade, we performed a dose-response experiment in which the antinatriuretic and antidiuretic responses to acute administration of the mineralocorticoid DOCA were investigated. Experiments were performed in chronically instrumented rats treated with intravenous canrenoate. Untreated rats were used as controls. After 2-wk treatment with canrenoate, the animals were transferred to a restraining cage, and intravenous infusion (150 mM glucose, 13 mM sodium chloride, and 3 mM lithium chloride; 2.5 ml/h) with [3H]inulin (batch nos. 145 and 147; specific activity, 48.5 and 42.5 GBq/mmol, respectively; infusion rate 3.5 µCi/h; Amersham, Buckinghamshire, UK) was started. After a 90-min equilibration period, urine was collected during 30-min control periods. Next, intravenous infusion of the mineralocorticoid DOCA was started (prime: 0.4 µg/kg body wt; 8 µg·kg·1·h⁻¹·iv). Collections were made in one 60-min period followed by four 30-min periods.

Experimental groups. The following groups of animals were studied: sham (n = 6), sham-operated rats; sham-CAN (n = 6), sham-operated rats chronically treated with canrenoate (20 mg/24 h); CBL (n = 6), CBL rats; and CBL-CAN (n = 6), CBL rats chronically treated with canrenoate (20 mg/24 h). Within each group, an additional six to eight rats were used for immunoblotting analysis to determine AQP2 expression levels (see Membrane fractionation for immunoblotting).

Metabolism studies. During the last 5 days before the renal function study, rats were housed in metabolic cages (Techniplast, model 1700; Scandinur, Lellinge, Denmark) that allowed accurate determination of 24-h urine volume and food and water intake. Daily sodium balance was calculated as sodium intake minus urinary sodium excretion. To optimize urinary recovery of sodium, the metabolic cage was rinsed with 40–50 ml of demineralized water after every urine collection. During housing in metabolic cages, the diet was changed to a granulated standard diet (Altromin catalogue no. 1310; Altromin International) to which lithium citrate was added (12 mmol lithium/kg dry diet). This dose of lithium chloride produced plasma lithium concentrations in the range 0.1–0.2 mmol/l without influencing renal function (22). After 2 days of adaptation, daily sodium balance was measured during the last 3 days before the renal function study.

Renal clearance study. Renal function was examined by clearance techniques 5 wk after CBL or sham-CBL. Before the renal clearance experiments, all rats were adapted to the restraining cage used for these experiments by training them for two periods of 2 h each. To examine the rats at the same level of hydration, all experiments were started at 9:00 AM. The animal was transferred to a restraining cage, and intravenous infusion (150 mM glucose, 13 mM sodium chloride, 3 mM lithium chloride; 2.5 ml/h) with [3H]inulin (batch nos. 145 and 147; specific activity, 48.5 and 42.5 GBq/mmol, respectively; infusion rate 3.5 µCi/h; Amersham) and [3H]tetraethylammonium bromide (lot no. 2957–517; specific activity 0.10 GBq/mmol; infusion rate 1.5 µCi/h; New England Nuclear, Boston, MA) was started. After a 90-min equilibration period, urine was collected during two 30-min control periods. Next, intravenous infusion of the selective V₂-receptor antagonist OPC-31260 was started (prime: 400 µg·kg⁻¹·h⁻¹; Otsuka America Pharmaceuticals; see Ref. 54). This dose of OPC-31260 was chosen based on dose-response experiments that demonstrated that 800 µg·kg⁻¹·h⁻¹ produced a diuretic response that was ~90% of
the maximal response to OPC-31260, and, since higher doses caused sedation, this dose was used. Total body water content was kept constant during V2-receptor blockade by intravenous replacement of urine losses with 150 mM glucose. Volume replacement was performed as described earlier by use of a computer-driven servo-control system written in LabView (National Instruments, Austin, TX) and was developed in collaboration with Bie Data (Copenhagen, Denmark; see Refs. 3 and 18). Urine collections were made in one 60-min period followed by three 30-min periods. A steady-state diuresis was achieved 45–60 min after the onset of the OPC-31260 infusion. Arterial blood samples of 300 µl each were collected in ammonium-heparinized capillary tubes at the end of the equilibration period, at the end of the control period, 1 h after OPC-31260 administration was started, and at the end of the experiment. At the beginning of the equilibration period (i.e., at 9:00 AM), a 0.2-ml blood sample was collected for measurement of plasma sodium and potassium concentrations and plasma osmolality, and, for measurement of the plasma concentration of vasopressin, a 1.0-ml blood sample was collected in a prechilled test tube with 20 µl of 0.5 M EDTA, pH 7.4, and 10 µl of 20 × 10⁻³ M aprotinin. After centrifugation at 4°C, plasma was transferred to a prechilled test tube and was stored at −20°C for later determination. All blood samples were replaced immediately with heparinized blood from a normal donor rat.

During the clearance experiment, mean arterial pressure (MAP) and heart rate (HR) were measured continuously using Baxter Uniflow pressure transducers (Bentley Laboratories, Uden, Holland) connected to pressure and HR couplers (Hugo Sachs, Hugstetten, Germany) and were sampled online using a data-acquisition program written in LabView (National Instruments) and developed in collaboration with Bie Data. After the clearance experiment, all catheters were sealed, the bladder was flushed with ampicillin (0.6 mg/ml), and the animals were returned to their home cages. Two days later, an additional 800-µl blood sample was drawn in a prechilled test tube for measurements of the plasma aldosterone concentration. The blood sample was centrifuged immediately at 4°C, and plasma was transferred to a prechilled test tube and stored at −20°C until analysis. An additional 0.1-ml arterial blood sample was drawn for analysis of plasma bilirubin and alanine aminotransaminase (ALT), and then the rats were killed.

Analytic procedures. Urine volume was determined gravimetrically. Concentrations of sodium, potassium, and lithium in plasma and urine were determined by atomic absorption spectrophotometry using a Perkin-Elmer (Allerød, Denmark) model 2380 atomic absorption spectrophotometer. Urine and plasma osmolality were determined by use of a cryometric osmometer (model 3 Cl1; Advanced Instruments, Needham Heights, MA). [³H]Julinin and [¹⁴C]tetrathyrammonium bromide in plasma and urine were determined by dual-label liquid scintillation counting on a Packard Tri-Carb liquid scintillation analyzer (model 2250CA; Packard Instruments, Greve, Denmark). Plasma concentrations of bilirubin and ALT were measured by reflometry using a Reflotron (Boehringer, Mannheim, Germany). The plasma concentration of aldosterone was measured by RIA using a commercial kit (Coat-A-Count Aldosterone; DPC, Los Angeles, CA). Vasopressin was extracted from plasma on C₁₈ Sep-Pak cartridges and was measured by RIA, as described earlier (20).

Membrane fractionation for immunoblotting. An additional series of rats was prepared for immunocytochemical examination (n = 7–8 in all groups). The rats were anesthetized with halothane-nitrous oxide, and the right kidney was removed and immediately frozen in liquid nitrogen and stored at −80°C before analysis. The kidneys were homogenized (0.3 M sucrose, 25 mM imidazole, 1 mM EDTA (pH 7.2), 8.5 µM leupetin, and 1 mM phenylmethylsulfonyl fluoride), and the homogenates were centrifuged at 4,000 g for 15 min. Next, the supernatant was centrifuged at 200,000 µg for 1 h to produce a pellet containing both plasma membrane and intracellular vesicle fractions (26, 28). Gel samples were prepared using Laemmli sample buffer containing 2% SDS.

Electrophoresis and immunoblotting. Samples of membrane fractions (−2 µg/lane) were run on 12% polyacrylamide minigels (Bio-Rad Mini Protean II). For each gel, an identical gel was run in parallel and subjected to Coomassie staining to assure identical loading (45). The other gel was subject to immunoblotting. Blots were blocked with 5% milk in 80 mM Na₂HPO₄, 20 mM NaCl, 100 mM NaCl, and 0.1% Tween 20, pH 7.5, for 1 h and were incubated with affinity-purified anti-AQP2 (40 ng IgG/µl IgG; see Refs. 9 and 28–30). The labeling was visualized with horseradish peroxidase-conjugated secondary antibody (diluted 1:3,000; P448; Dako) using an enhanced chemiluminescence (ECL) system (Amersham). Controls were prepared with replacement of the primary antibody with an antibody preabsorbed with immunizing peptide IgG or with nonimmune IgG.

Quantification of AQP2 expression. ECL films with bands within the linear range were scanned (28) using a Hewlett-Packard Scanjet scanner. For AQP2, both the 29-kDa and the 35–50-kDa bands corresponding to the nonglycosylated and the glycosylated species (40) were scanned as described earlier (16, 26, 28, 45). The labeling density was quantitated (26, 28) from blots from canrenoate-treated rats and untreated CBL rats run on a gel along with control material taken from untreated sham-operated animals. AQP2 labeling in samples from the canrenoate-treated rats and the untreated cirrhotic rats was expressed relative to the mean expression in the corresponding control material run on the same gel.

Preparation of RNA samples and Northern blotting. Total RNA was extracted from whole kidney from untreated and canrenoate-treated sham rats (n = 6 in both groups) using the acid guanidium-isothiocyanate-phenol-chloroform method (6). Quantification of AQP2 message was performed using a digoxigenin-labeled AQP2 RNA probe (7). The synthesis and digoxigenin labeling of AQP2 RNA probe were performed by in vitro transcription using a Maxiscript in vitro transcription kit (Ambion, Austin, TX). RNA samples (7 µg) were denatured and separated by electrophoresis on a gel agarose (1.2%) containing 0.6 M formaldehyde. Equal RNA loading was verified by visual inspection after coloration with ethidium bromide. The RNA were transferred overnight from gel to nylon membranes (Hybond-N; Amersham Life Science) that were then baked in a vacuum oven (2 h at 80°C). Blots were placed in a glass hybridization tube containing 5× saline sodium citrate (SSC), 50% formamide, 0.1% sarkosyl, 0.02% SDS, and 2% blocking solution (blocking reagent in maleic acid; Boehringer). Prehybridization was performed at 55°C for 30 min in a hybridization oven. The digoxigenin-labeled AQP2 RNA probe was then added to prehybridization medium, and membranes were incubated overnight at 55°C. The blots were washed two times at 25°C in 2× SSC and 0.1% SDS for 5 min and two times at 68°C in 0.1× SSC and 0.1% SDS for 15 min. Blots were then equilibrated for 1 min in maleic acid solution containing 0.3% Tween 20 and were blocked for 30 min. After incubation for 30 min with anti-digoxigenin-alkaline phosphatase conjugate (Boehringer), blots were washed two times for 15 min in maleic acid solution containing 0.3% Tween 20 and were equilibrated for 5 min in...
Lithium clearance (CLi) was used as a marker for the outflow effective renal plasma flow (ERPF; see Ref. 34). Ethylammonium clearance was used as a marker for the inulin clearance was used as a marker for GFR, and tetraethylammonium clearance was used as a marker for the effective renal plasma flow (ERPF; see Ref. 34).

The effective filtration fraction (EFF) was calculated as

\[ \text{EFF} = \frac{\text{GFR}}{\text{ERPF}} \]

Lithium clearance (CLi) was used as a marker for the outflow of tubular fluid from the proximal tubules (46). Thus CLi/GFR is an estimate of the fractional delivery of fluid and sodium from the proximal tubules, and V/CLi represents the fractional distal water excretion.

Micropuncture studies on the effect of furosemide on tubular lithium handling suggest that, during control conditions, 2–5% of filtered lithium may be reabsorbed in the TAL, and therefore only changes of the fractional excretion of lithium (FE Li) in excess of 2–5% can be attributed to changes in proximal tubular sodium reabsorption (15, 43). However, when comparisons are performed between groups in which all animals are treated with furosemide, any difference among groups can be ascribed to changes in proximal tubular sodium reabsorption, since there is no evidence for lithium reabsorption beyond the early distal convoluted tubules in sodium-replete rats (15, 35).

Statistics. Data are presented as means ± SE. To evaluate the effects of V2-receptor blockade, the average value during the two 30-min control periods was compared with the average value during the last two 30-min periods during OPC-31260-induced diuresis. Within-group comparisons were analyzed with Student’s paired t-test. Between-group comparisons were performed by one-way ANOVA followed by Fisher’s least-significant difference test. Differences were considered significant at the 0.05 level.

RESULTS

Efficacy of aldosterone-receptor blockade. In untreated rats, acute DOCA infusion induced a significant fall in fractional sodium and lithium excretion without changes in GFR. This antinatriuresis and antilihiuresis were completely absent in rats treated with 20 mg canrenoate/day. Because higher doses of canrenoate caused hyperkalemia and decreased the daily weight gain in normal rats, we chose the dose of 20 mg canrenoate/day (Fig. 1).

Effect of canrenoate on plasma biochemistry and plasma osmolality. Chronic aldosterone-receptor blockade had no effect on the plasma level of vasopressin, which was significantly increased in the cirrhotic rats.
aldosterone-receptor blockade induced a significant increase in solute-free urine.

In accordance with previous studies (19), daily sodium intake was similar in all groups, but daily sodium excretion was significantly decreased in untreated cirrhotic rats, which caused sodium retention relative to control animals. Despite the well-described effect of aldosterone-receptor blockade on the CD reabsorption, canrenoate treatment had no significant effect on sodium excretion in cirrhotic rats, which caused sodium retention relative to control animals. Despite the well-described effect of aldosterone-receptor blockade on the CD reabsorption, canrenoate prevented sodium retention due to a significant 22% increase in the daily sodium excretion (Table 1). However, the aquarectic effect of OPC-31260 was significantly attenuated in normal rats with chronic aldosterone-receptor blockade: $\Delta V, -29\% (57 \pm 8 \text{ vs. } 81 \pm 4 \mu l/min/100 \text{ g}, P < 0.01); \Delta C_{\text{H}_2\text{O}}, -29\% (59 \pm 8 \text{ vs. } 83 \pm 5 \mu l/min/100 \text{ g}, P < 0.01); \text{ and } \Delta V/C_{\text{Li}}, -26\% (22 \pm 2 \text{ vs. } 29 \pm 3\%, P < 0.01).$ Thus there is a marked reduction in vasopressin-dependent water reabsorption in response to canrenoate treatment. To test if this also was the case in rats with CBL-induced cirrhosis, cirrhotic rats were subjected to combined canrenoate and OPC-3160 treatment. Cirrhotic rats had, as previously demonstrated (18), a significantly decreased aquarectic response to acute V2-receptor blockade: $\Delta V, -29\% (58 \pm 4 \text{ vs. } 81 \pm 4 \mu l/min/100 \text{ g}, P < 0.01); \Delta C_{\text{H}_2\text{O}}, -28\% (59 \pm 3 \text{ vs. } 83 \pm 5 \mu l/min/100 \text{ g}, P < 0.01); \text{ and } \Delta V/C_{\text{Li}}, -34\% (29 \pm 3 \text{ vs. } 19 \pm 1\%, P < 0.01).$ In canrenoate-treated cirrhotic rats, the aquarectic response to V2-receptor blockade was further impaired: $\Delta V, -21\% (45 \pm 3 \text{ vs. } 58 \pm 4 \mu l/min/100 \text{ g}, P < 0.05); \Delta C_{\text{H}_2\text{O}}, -32\% (40 \pm 5 \text{ vs. } 59 \pm 3 \mu l/min/100 \text{ g}, P < 0.05); \text{ and } \Delta V/C_{\text{Li}}, -32\% (13 \pm 2 \text{ vs. } 19 \pm 1\%, P < 0.05).$

Renal expression of AQP2 protein. Figures 4 and 5 show immunoblots of membrane fractions (2 µg/ml) from whole kidney preparations. As previously shown, the affinity-purified anti-AQP2 protein antibody recognizes the 29-kDa and the 35- to 50-kDa band, corresponding to nonglycosylated and glycosylated AQP2 protein, respectively. As shown in Fig. 4A, a significant decrease of both the 29-kDa and the 35- to 50-kDa AQP2 bands was observed in normal rats chronically treated with canrenoate. Densitometry of all samples (Fig. 4B) from canrenoate-treated normal rats revealed a 45% decrease in AQP2 expression (sham-CAN: 55 ± 10% vs. sham: 100 ± 12%, P < 0.01). Cirrhotic rats had, as shown in Fig. 5A, a significant decrease in labeling of both the 29-kDa and the 35- to 50-kDa AQP2 bands, and densitometry of all samples (Fig. 5B) revealed a 86% decrease in AQP2 expression (CBL, 14 ± 3% vs. sham, 100 ± 12%, P < 0.01). Canrenoate treatment did not induce any further measurable downregulation in AQP2 levels in cirrhotic rats (CBL-CAN, 78 ± 17% vs. CBL, 100 ± 12%, not significant). Thus the polypuria and the decreased response to acute vasopressin V2-receptor blockade during chronic aldosterone-receptor blockade was associated with a marked decrease in AQP2 expression, both in normal and cirrhotic rats.
Table 2. Daily water intake, urine production, urine osmolality, sodium intake, sodium excretion, and sodium balance 4–5 wk after CBL or sham in untreated rats and in rats chronically treated with the aldosterone-receptor antagonist canrenoate

<table>
<thead>
<tr>
<th>Daily Water Intake, ml·day⁻¹·100 g body wt⁻¹</th>
<th>Daily Urine Production, ml·day⁻¹·100 g body wt⁻¹</th>
<th>Daily Urine Osmolality, mosmol/kgH₂O</th>
<th>Daily Sodium Intake, mmol/day</th>
<th>Daily Sodium Excretion, mmol/day</th>
<th>Daily Sodium Balance, mmol/day</th>
</tr>
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<tbody>
<tr>
<td>Sham</td>
<td>15.2 ± 2.1</td>
<td>7.7 ± 0.9</td>
<td>1261 ± 107</td>
<td>2.59 ± 0.13</td>
<td>0.39 ± 0.07</td>
</tr>
<tr>
<td>Sham-CAN</td>
<td>19.0 ± 1.0†</td>
<td>11.1 ± 0.9*</td>
<td>919 ± 94*</td>
<td>2.62 ± 0.25</td>
<td>0.26 ± 0.13</td>
</tr>
<tr>
<td>CBL</td>
<td>15.4 ± 1.5</td>
<td>7.9 ± 0.7</td>
<td>1314 ± 96</td>
<td>2.66 ± 0.11</td>
<td>0.77 ± 0.14*</td>
</tr>
<tr>
<td>CBL-CAN</td>
<td>23.0 ± 3.1†‡</td>
<td>15.3 ± 2.8†</td>
<td>807 ± 137†‡</td>
<td>2.67 ± 0.17</td>
<td>0.36 ± 0.12†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 13 rats in each group. Dose of canrenoate used was 20 mg/day iv. Daily sodium balance was calculated as sodium intake minus urinary sodium excretion. *P < 0.05 vs. sham. †P < 0.05 vs. CBL.

Effect of canrenoate on renal expression of AQP2 mRNA. Figure 6 shows a Northern blot of AQP2 mRNA in total kidney of normal rats either untreated or treated with canrenoate. Figure 6B shows a digoxigenin-labeled rat AQP2 RNA probe of ~1.6 kb, consistent with the predicted size of AQP2 mRNA. Densitometry of all samples (Fig. 6C) from canrenoate-treated normal rats revealed a nonsignificant downregulation of the AQP2 mRNA expression in normal rats treated with canrenoate (sham-CAN, 67 ± 27% vs. sham, 100 ± 21%; not significant).

Canrenoate treatment had no effect on body and kidney weight in normal or cirrhotic rats (Table 4).

DISCUSSION

The present results demonstrate that chronic treatment with the aldosterone receptor-antagonist canrenoate (20 mg/day iv for 4 wk) significantly 1) increases urine production and decreases urine osmolality, 2) decreases the aquaretic effect of selective vasopressin V₂-Receptor blockade, and 3) decreases AQP2 protein abundance, in the absence of changes in plasma vasopressin levels. Together these results suggest that chronic treatment with the aldosterone antagonist canrenoate decreases vasopressin-mediated renal water reabsorption and increases the daily production of solute-free urine and that this involves downregulation of the CD water channel AQP2.

Changes in CD function during chronic aldosterone-receptor blockade. Conditions with adrenal insufficiency are characterized by an inability to generate a maximally concentrated urine (49). Similarly, adrenalectomized rabbits have an impaired urinary concentration ability that can be normalized by administration of either gluco- or mineralocorticoids (42). In vitro studies on isolated cortical CD from adrenalectomized rabbits showed that the vasopressin-stimulated increase in osmotic water permeability was impaired in adrenalectomized animals but could be restored by treatment with mineralocorticoids (42). Furthermore, acute or 4–14 days treatment with mineralocorticoids increased the vasopressin-mediated osmotic water permeability in isolated cortical CD in normal rabbits (5). In rats, this synergistic action of mineralocorticoid and vasopressin on osmotic water permeability was absent in isolated cortical CD from normal rats treated with mineralocorticoid for 4–8 days (5). However, Ray et al. (37) showed that the vasopressin-mediated osmotic water permeability was significantly impaired in papillary segments of CD from adrenalectomized rats 3 wk after adrenalectomy. Together, these observations strongly suggest that mineralocorticoids are involved in the regulation of the CD water permeability. The present results demonstrate that chronic aldosterone-receptor blockade inhibits the vasopressin-mediated water reabsorption in the absence of changes in the plasma vasopressin level in normal rats and in rats with liver cirrhosis. Moreover, the results suggest that downregulation of AQP2 plays a significant role in this. Theoretically, this effect could be due to a primary polydipsic effect of canrenoate causing secondary polyuria, but the lack of changes in plasma vasopressin strongly indicates that this is not the case. Therefore, the results suggest that mineralocorticoid receptor blockade downregulates AQP2 abundance in the CD and thereby the CD water permeability. The mechanisms behind this effect are unknown, but the lack of a significant downregulation of the AQP2 mRNA level (Fig. 6) could suggest that an increased degradation of the AQP2 protein was involved. Because plasma vasopressin levels are normal, vasopressin-independent mechanisms may play a role for this reduction in AQP2 expression. This will be discussed below.

Vasopressin regulates water permeability in the renal CD by short-term and long-term regulation. CD water permeability increases within a few minutes in response to an acute increase in plasma vasopressin concentration, and this is mediated by shuttling of AQP2 from intracellular vesicles into the apical plasma membrane via exocytosis (29, 30, 38, 54). For long-term regulation of body water, the total amount of AQP2 protein in the principal cells is increased (30) along with increased AQP2 mRNA levels (23) due, at least in part, to increased AQP2 gene transcription (32). Conversely, in the absence of vasopressin, e.g., in vasopressin-deficient Brattleboro rats, AQP2 expression is suppressed (9). From several studies, it has become clear that both vasopressin-dependent and vasopressin-independent mechanisms operate to modulate AQP2 expression levels (for recent review see Ref. 31). Long-term treatment of vasopressin-deficient Brattleboro rats with vasopressin resulted in 1) a marked increase in AQP2 expression levels, 2) increased osmotic water permeability of inner medullary CD, and 3) complete restoration of the urinary concentration defect (9). This directly demonstrated that vasopressin regulates AQP2
expression levels. The identification of a cAMP-response element in the 5’-flanking region of the AQP2 gene (47) is consistent with an important role of vasopressin V2 receptor-mediated increases in cAMP and cAMP-dependent protein kinase activity on AQP2 expression. The first indication that vasopressin-independent regulation may also be involved came from a study with rats having extremely severe nephrogenic diabetes insipidus due to chronic lithium treatment. Thirsting of such rats for 48 h produced a much greater increase in AQP2 expression than did 7 days of 1-desamino-8-D-arginine vasopressin (DDAVP) treatment (26). Subsequently, Ecelbarger and colleagues (11, 12) demonstrated that water loading of rats that had clamped high levels of plasma DDAVP levels (which prior to water loading increased AQP2 expression) escapes from the effect of DDAVP and produces a significant reduction in AQP2 levels. Recently, it was also demonstrated that thirsting of rats in the continued presence of chronic V2-receptor blockade (OPC-31260) markedly increased AQP2 expression levels (25). These studies together support the view that vasopressin-independent mechanisms may play a significant role in modulating AQP2 expression levels, and several studies suggest that this pathway may be involved in several water balance disorders.

Dysregulation of AQP2 expression has been shown to be associated with several diseases or conditions with severe disturbances in renal water and salt handling. Deen et al. (8) demonstrated that mutant, nonfunctional AQP2 was the cause of very severe non-X-linked inherited nephrogenic diabetes insipidus in humans, making it clear that AQP2 was essential for renal water conservation. Subsequently, it was demonstrated that downregulation of AQP2 expression and reduced targeting of AQP2 was associated with several forms of acquired nephrogenic diabetes insipidus, such as lithium treatment (26), hypokalemia (27), hypercalcemia (10, 39), and ureteral obstruction (16). Conversely, it was found that AQP2 expression is increased in rats with severe congestive heart failure associated with hyponatremia and increased plasma vasopressin levels (33, 52), and also pregnant rats with water retention (24) have been shown to have increased AQP2 expression levels. Thus dysregulation of AQP2 appears to be involved in many water balance disorders. The present study demonstrates that chronic aldosterone-receptor blockade is associated with a 45% reduction in AQP2 expression levels (Fig. 4) and a significant polyuria.
(44% increase, Table 2 and Fig. 2) in the absence of changes in plasma vasopressin levels. Thus this condition shares similarities with other forms of acquired nephrogenic diabetes insipidus with moderate polyuria and urinary concentrating defects such as hypokalemia, hypercalcemia, and postobstructive polyuria (as described above). All of these conditions are also associated with a 50–200% increase in urine production and 40–70% reduction in AQP2 expression levels. The demonstration of a significant attenuation of the aquarectic response to acute vasopressin V2-receptor blockade (with OPC-31260) in canrenoate-treated animals (Fig. 3) is consistent with the view that downregulation of AQP2 expression is likely to play a significant role in producing the polyuria (i.e., the defect in the CD; aldosterone-receptor blockade is also likely to induce
kidney-to-body weight ratio. As previously shown (15, 16, 17), renal hypertrophy and increased treatment had no effect on body or kidney weight. Cirrhotic rats had, significantly.

Samples. Canrenoate treatment reduced AQP2 mRNA nonsignificantly. densitometric analysis of expression of AQP2 mRNA from all C, a band of 1.6 kb, consistent with the predicted size of AQP2 mRNA. Fig. 6: Northern blot of AQP2 mRNA in total kidney of rats treated with canrenoate or from sham-operated/treated rats (sham). A: as a loading control, ribosomal 18S and 28S rRNA bands were visualized by ethidium bromide on the same gel used for blotting and hybridization (shown in B). B: digoxigenin-labeled rat AQP2 RNA probe labels a band of ~1.6 kb, consistent with the predicted size of AQP2 mRNA.

C: densitometric analysis of expression of AQP2 mRNA from all samples. Canrenoate treatment reduced AQP2 mRNA nonsignificantly.

effects in other tubule segments, see below). This reduction in AQP2 expression occurred in the absence of changes in plasma vasopressin levels. Thus this is also similar to other forms of acquired nephrogenic diabetes insipidus in which plasma vasopressin levels are unchanged or perhaps even increased, indicating that vasopressin-independent regulation (or dysregulation) of AQP2 may be involved in these conditions. The present study raises the possibility that modulation of aldosterone receptor activation may play a significant role in regulating (or maintaining) AQP2 expression. However, further studies are required to fully define the general importance of this in the physiology of water balance and pathophysiology of water balance disorders.

Changes in TAL function during chronic aldosterone-receptor blockade. The TAL plays a major role in the renal concentration mechanism. A number of hormones, including vasopressin, stimulate sodium reabsorption in the TAL (41, 50). In addition to the well-known stimulatory effect of aldosterone on sodium reabsorption in the CD, studies using in vivo perfusion of Henle’s loop of superficial nephrons (44) and in vitro perfusion of isolated TAL (51) have shown that aldosterone-replacement therapy normalizes the decreased TAL sodium reabsorption in adrenalectomized rats. We recently demonstrated that rats with compensated liver cirrhosis have increased furosemide-sensitive sodium chloride reabsorption and tubular hypertrophy of the TAL. These functional and structural changes are associated with sodium retention and an increased interstitial sodium concentration in the renal medulla (17, 18). As a consequence of the increased corticopapillary interstitial osmotic gradient, the driving force for non-vasopressin-mediated water reabsorption is increased in cirrhotic rats. This likely explains why cirrhotic rats, despite a significant downregulation of AQP2 expression and an attenuated diuretic response to selective V2-receptor blockade, had a normal daily urine production. Thus the downregulation of AQP2 may be compensatory to avoid water retention, in similarity to the downregulation of AQP2 seen in water-loaded, DDAVP-treated rats (11, 12), thereby preventing water intoxication. In the present study, chronic aldosterone-receptor blockade increased the daily urine production by 92% in cirrhotic rats compared with 44% in normal rats. The response to acute V2-receptor blockade was blunted to the same extent as in the normal rats, but the immunoblotting did not show any further significant downregulation of AQP2 in the canrenoate-treated cirrhotic rats. We have recently shown that canrenoate inhibits the increased furosemide-sensitive sodium reabsorption in the TAL in cirrhotic rats (19). These data suggest that canrenoate, at least in cirrhotic rats, decreases sodium chloride reabsorption in the TAL and thereby impairs the corticopapillary interstitial gradient. Thus, in cirrhotic rats, canrenoate also decreases the driving force for transepithelial water reabsorption across the CD and increases urine flow rate. Therefore, inhibition of an increased sodium chloride reabsorption in the TAL in cirrhotic rats may explain why canrenoate produced a significantly greater increase in 24-h urine production in cirrhotic rats than in normal animals.

In summary, chronic treatment with the aldosterone-receptor antagonist canrenoate (20 mg/day iv for 4 wk)
significantly 1) increases urine production and decreases urine osmolality, 2) decreases the aquaretic effect of selective V2-receptor blockade, and 3) decreases the AQP2 protein abundance, and this occurs in the absence of changes in plasma vasopressin levels. Together these results suggest that chronic treatment with the aldosterone antagonist carnovento decreases vasopressin-mediated renal water reabsorption, decreases CD AQP2 expression, and increases the daily production of solute-free urine. These findings support the view that aldosterone-receptor antagonists may be particularly effective drugs during conditions with avid vasopressin-mediated water retention and hyponatremia.

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