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

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Jonassen, Thomas E. N., Dominique Promeneur, Sten Christensen, Jørgen S. Petersen, and Søren Nielsen. Decreased vasopressin-mediated renal water reabsorption in rats with chronic aldosterone-receptor blockade. Am. J. Physiol. Renal Physiol. 278: F246–F256, 2000.—Previous studies have suggested that mineralocorticoids are needed for a normal action of vasopressin on collecting duct osmotic water permeability. However, the mechanisms behind this are unknown. To investigate if aldosterone-receptor blockade influences vasopressin type 2 receptor (V$_2$)–mediated renal water reabsorption and the renal expression of the vasopressin–regulated water channel aquaporin-2 (AQP2), rats were treated with the aldosterone-receptor antagonist canrenoate (20 mg/day iv) for 4 wk. Daily urine flow was increased significantly by 44%, and urine osmolality was decreased by 27% in canrenoate-treated rats. Acute V$_2$-receptor blockade (OPC-31260, 800 µg·kg$^{-1}$·h$^{-1}$) was performed under conditions in which volume depletion was prevented. In control rats, OPC-31260 induced a significant increase in urine flow rate (V$_f$ +25%) and free water clearance (C$_{H$_2$O}$, −29%). In canrenoate-treated rats, the effect of OPC-31260 was significantly reduced, and semiquantitative immunoblotting demonstrated a significant reduction (45%) in AQP2 expression. Because rats with common bile duct ligation (CBL) have a reduced vasopressin-mediated water reabsorption compared with normal rats (V$_f$ −24%; C$_{H$_2$O}$, −28% and 86% downregulation of AQP2), the effect of canrenoate combined with OPC-31260 was tested. Canrenoate treatment of CBL rats significantly increased daily urine flow, decreased urine osmolality, and impaired the aquaretic response to OPC-31260 (V$_f$ −23%; C$_{H$_2$O}$, −31%) with maintained suppression of the renal AQP2 expression. Thus canrenoate treatment of normal and CBL rats showed 1) increased urine production, 2) reduced aquaretic effect of acute V$_2$-receptor blockade, and 3) a marked reduction in AQP2 expression. This strongly supports the view that aldosterone plays a significant role for vasopressin-mediated water reabsorption.

aquaporin-2; V$_2$-receptor; OPC-31260; collecting ducts; canrenoate; cirrhosis

Chronic adrenal insufficiency is characterized by an inability 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 diuretic order 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 microperfusion studies of Henle’s loop and in vitro studies on isolated segments of the thick ascending limb of Henle’s (TAL) 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 (V$_2$ 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 (28, 30, 38, 53). During prolonged increases in plasma vasopressin levels, the AQP2 expression is markedly increased (30). However, mechanisms other than vasopressin seem to be involved in the long-term regulation of AQP2 expression. AQP2 expression is increased in pregnant rats with normal plasma vasopressin levels (24). Moreover, downregulation of renal AQP2 protein levels has been reported in a number of conditions with normal or increased plasma vasopressin levels like nephrotic syndrome (1, 14), hypokalemia (27), hypercalcemia (10, 39), ureteral obstruction (16), and compensated liver cirrhosis (18).

An increased plasma aldosterone level is considered among the most important mechanisms involved in the
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, V2-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 V2-receptor blockade was induced by intravenous administration of the selective V2-receptor antagonist OPC-31260. V2-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 (Nuclear, Boston, MA) 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: 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; Scandibur, 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 allowed the diet to produce plasma lithium levels 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 remaining 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 [\(^{3}H\)]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 two 30-min control periods. Next, intravenous infusion of the selective V2-receptor antagonist OPC-31260 was started (prime: 400 µg/kg body wt; 800 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
was kept constant during V2-receptor blockade by intrave-
causa sedation, this dose was used. Total body water content
the maximal response to OPC-31260, and, since higher doses
case a steady-state diuresis was achieved 45–60 min after the onset of
the control period, 1 h after OPC-31260 administration was started, and
by 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 Labora-
tories, Uden, Holland) connected to pressure and HR couplers
(Hugo Sachs, Hugstetten, Germany) and were sampled on-
line 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 and was stored at
20°C for later
determination. All blood samples were replaced immediately
with heparinized blood from a normal donor rat.

Analytic procedures. Urine volume was determined gravi-
metrically. Concentrations of sodium, potassium, and lithium
in plasma and urine were determined by atomic absorption
spectrophotometry using a Perkin-Elmer Aanal 8000 (Perkin-Elmer, Denmark). Plasma osmolality and sodium
were determined by use of a cryometric osmometer (model 3 CI; Advanced Instruments, Needham Heights, MA). [3H]Julin and [14C]etraethylammonium 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 alanine aminotransaminase (ALT), and membranes were incubated with affinity-purified anti-AQP2 (40 ng IgG/µl IgG; see Refs. 9 and 28–30). The labeling density was quantitated
(26, 28) from blots from canrenoate-treated rats and un-
treated 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 un-
treated cirrhotic rats was expressed relative to the mean
density 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
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% sarscyl, 0.02% SDS, and 2% blocking solution (blocking reagent in malec 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 me-
dium, and membranes were incubated overnight at 55°C. The
blots were washed twice 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 malec acid solution
containing 0.3% Tween 20 and were equilibrated for 5 min in

Membrane fractionation for immunoblotting. An additional
series of rats was prepared for immunocytochemical examina-
tion (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
leupeptin, and 1 mM phenylmethylsulfonfyl fluoride], and
the homogenates were centrifuged at 4,000 g for 15 min. Next,
the supernatant was centrifuged at 200,000 × g 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 mem-
brane fractions (~2 µg/lane) were run on 12% polyacrylamide
minigels (Bio-Rad Mini Protein 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 subjected
to immunoblotting. Blots were blocked with 5% milk in 80 mM
Na2HPO4, 20 mM NaH2PO4, 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).

Quantitation of AQP2 expression. ECL films with bands
within the linear range were scanned (28) using a Hewlett-
Packard ScanJet et scanner. For AQP2, both the 29-kDa and the
35- to 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
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in samples from the canrenoate-treated rats and the un-
treated cirrhotic rats was expressed relative to the mean
density in the corresponding control material run on the
same gel.
Lithium clearance ($CL_i$) 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

$$EFF = \frac{GFR}{ERPF}$$

Lithium clearance ($C_{Li}$) was used as a marker for the outflow of tubular fluid from the proximal tubules (46). Thus $C_{Li}/GFR$ is an estimate of the fractional delivery of fluid and sodium from the proximal tubules, and $V/C_{Li}$ 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 normal 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 antilithiuresis 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.
Canrenoate treatment 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 the daily sodium excretion in normal rats when investigated during the fourth week of canrenoate treatment. This indicates that a new steady state in sodium balance was reached after an initial sodium loss in the first week of treatment. However, in cirrhotic rats, canrenoate prevented sodium retention due to a significant 22% increase in the daily sodium excretion (Table 2 and Fig. 2). This indicates that canrenoate significantly inhibited an increased tubular sodium reabsorption in cirrhotic rats.

Effect of canrenoate on systemic and renal hemodynamics and fractional lithium excretion. Table 3 shows baseline levels of systemic and renal hemodynamics and renal lithium handling during the clearance experiments performed after 4 wk intravenous treatment with canrenoate. Canrenoate had no effect on MAP (which was significantly decreased in the cirrhotic rats), GFR, or FE\textsubscript{Li}. However, canrenoate treatment normalized the increased renal plasma flow (ERPF) and the decreased filtration fraction (EFF) found in the cirrhotic rats. Acute V\textsubscript{2}-receptor blockade had, as previously shown (18), no effect on MAP, ERPF, GFR, EFF, or FE\textsubscript{Li} (data not shown).

Effect of V\textsubscript{2}-receptor antagonist OPC-31260 on renal water handling. Acute intravenous treatment with the V\textsubscript{2}-receptor antagonist OPC-31260 significantly increased urine flow rate, free water clearance (C\textsubscript{H\textsubscript{2}O}), and V/C\textsubscript{Li} in all four groups (Fig. 3). However, the aquaretic effect of OPC-31260 was significantly attenuated in normal rats with chronic aldosterone-receptor blockade: \( \Delta V, -29\% \) (57 ± 8 vs. 81 ± 4 \( \mu \text{ml} \cdot \text{min}^{-1} \cdot \text{100 g}^{-1} ; P < 0.01); \( \Delta C_{\text{H}_{2}\text{O}} \), -29\% (59 ± 8 vs. 83 ± 5 \( \mu \text{ml} \cdot \text{min}^{-1} \cdot \text{100 g}^{-1} ; P < 0.01); and \( \Delta V/C_{\text{Li}} \), -26\% (22 ± 2 vs. 29 ± 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-31260 treatment. Cirrhotic rats had, as previously demonstrated (18), a significantly decreased aquaretic response to acute V\textsubscript{2}-receptor blockade: \( \Delta V, -29\% \) (58 ± 4 vs. 81 ± 4 \( \mu \text{ml} \cdot \text{min}^{-1} \cdot \text{100 g}^{-1} ; P < 0.01); \( \Delta C_{\text{H}_{2}\text{O}} \), -28\% (59 ± 3 vs. 83 ± 5 \( \mu \text{ml} \cdot \text{min}^{-1} \cdot \text{100 g}^{-1} ; P < 0.01); and \( \Delta V/C_{\text{Li}} \), -34\% (29 ± 3 vs. 19 ± 1%; P < 0.01). In canrenoate-treated cirrhotic rats, the aquaretic response to V\textsubscript{2}-receptor blockade was further impaired: \( \Delta V, -21\% \) (45 ± 3 vs. 58 ± 4 \( \mu \text{ml} \cdot \text{min}^{-1} \cdot \text{100 g}^{-1} ; P < 0.05); \( \Delta C_{\text{H}_{2}\text{O}} \), -32\% (40 ± 5 vs. 59 ± 3 \( \mu \text{ml} \cdot \text{min}^{-1} \cdot \text{100 g}^{-1} ; P < 0.05); and \( \Delta V/C_{\text{Li}} \), -32\% (13 ± 2 vs. 19 ± 1%; P < 0.05).

Renal expression of AQP2 protein. Figures 4 and 5 show immunoblots of membrane fractions (2 \( \mu \text{g} \)/lane) 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 polyuria and the decreased response to acute vasopressin V\textsubscript{2}-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></th>
<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/kg H2O</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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<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>2.20 ± 0.18</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>2.36 ± 0.13</td>
<td>0.26 ± 0.04</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>1.89 ± 0.18</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>2.31 ± 0.23†</td>
<td>0.36 ± 0.12†</td>
</tr>
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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 mineralocorticoid 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 polypsipic 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.
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.

Table 3. Effect of 4-wk treatment with canrenoate on systemic and renal hemodynamics and renal lithium handling

<table>
<thead>
<tr>
<th></th>
<th>MAP, mmHg</th>
<th>ERPF, ml·min⁻¹·100 g body wt⁻¹</th>
<th>GFR, ml·min⁻¹·100 g body wt⁻¹</th>
<th>EFF, %</th>
<th>FE Li, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>118±4</td>
<td>3.83±0.33</td>
<td>1.04±0.06</td>
<td>27.2±0.4</td>
<td>24.2±1.6</td>
</tr>
<tr>
<td>Sham-CAN</td>
<td>112±3</td>
<td>3.93±0.28</td>
<td>0.95±0.03</td>
<td>24.1±1.0</td>
<td>28.4±2.3</td>
</tr>
<tr>
<td>CBL</td>
<td>105±2*</td>
<td>5.05±0.40*</td>
<td>1.00±0.07</td>
<td>20.6±2.1*</td>
<td>25.1±2.3</td>
</tr>
<tr>
<td>CBL-CAN</td>
<td>108±2*</td>
<td>4.23±0.35</td>
<td>0.97±0.08</td>
<td>23.5±2.3</td>
<td>27.7±2.3</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 rats in each group. Results are from baseline conditions during renal clearance experiments 5 wk after CBL or sham. MAP, mean arterial pressure; ERPF, effective renal plasma flow; GFR, glomerular filtration rate; EFF, effective filtration fraction; FE Li, fractional lithium clearance. *P < 0.05 vs. sham.
(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 aquaretic 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
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 canrenoate 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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