Renal Expression of Aquaporins in Liver Cirrhosis Induced by Chronic Common Bile Duct Ligation in Rats

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Abstract. Semiquantitative immunoblotting was used to investigate the expression levels of the four major renal aquaporins, the Na-K-2Cl cotransporter of the thick ascending limb, the type 3 Na-H exchanger, and the Na-K-ATPase in kidneys from rats with cirrhosis secondary to common bile duct ligation (CBDL). These rats had significant water retention and hyponatremia. In contrast to models of cirrhosis induced by carbon tetrachloride, aquaporin-2 expression in CBDL-induced cirrhosis was decreased. Thus, these results show that in the setting of extracellular fluid volume expansion, excessive water retention with hyponatremia can occur in the absence of increases in aquaporin-2 abundance. In addition, the expression levels of the two basolateral collecting duct aquaporins (aquaporin-3 and -4) were decreased in CBDL rats relative to sham-operated control rats. Similarly, the Na-K-2Cl cotransporter of the thick ascending limb and the type 3 Na-H exchanger showed decreases in expression. In contrast, the expression levels of aquaporin-1 and the α1 subunit of the Na-K-ATPase were not decreased. Thus, dysregulation of multiple water channels and ion transporters may play a role in water balance abnormalities associated with CBDL-induced cirrhosis in rats.

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Osmotic water reabsorption in the renal collecting ducts is mediated by water channels called aquaporins (1,2). Three aquaporins are expressed in the mammalian collecting duct: aquaporins-2, -3, and -4. Aquaporin-2 mediates water transport across the apical plasma membranes of collecting duct principal cells, and aquaporins-3 and -4 mediate basolateral transport. Aquaporin-2 is regulated by vasopressin in two ways: short-term regulation by stimulation of trafficking of aquaporin-2-containing vesicles to the apical plasma membrane and long-term regulation through augmentation of total aquaporin-2 protein abundance in the collecting duct cells. Aquaporin-3 abundance appears to be regulated on a long-term basis in a manner similar to the long-term regulation of aquaporin-2. However, aquaporin-4 is not known to be regulated by vasopressin.

The analysis of water retention in pathophysiologic states associated with extracellular fluid volume expansion (congestive heart failure, nephrotic syndrome, and cirrhosis) has focused mainly on aquaporin-2. Severe congestive heart failure with hyponatremia, induced by coronary artery ligation in rats, was associated with increased aquaporin-2 abundance in collecting duct principal cells (4,5). This response was attributed to high circulating levels of vasopressin secondary to nonosmotic stimulation of vasopressin release (4). In contrast, nephrotic syndrome induced in rats by administration of puromycin aminonucleoside manifested decreased aquaporin-2 expression despite high circulating levels of vasopressin (6). Similar decreases in aquaporin-2 expression were seen in adriamycin-induced nephrotic syndrome in rats (7,8). Initial observations in rats with CCl4-induced cirrhosis showed a moderate increase in aquaporin-2 expression (9,10). However, subsequent studies with cirrhosis induced in rats by common bile duct ligation (CBDL) (11) did not show increases in aquaporin-2. In fact, with CBDL, aquaporin-2 expression was decreased (11). Thus, the findings with regard to aquaporin-2 expression are not uniform among the physiologic states or even among different experimental models nominally of the same disease process. In addition to the collecting duct, the loop of Henle also plays a vital role in the regulation of water excretion. In this article, we investigated the effect of CBDL-induced cirrhosis on the expression of all three collecting duct aquaporins, as well as on the expression of two proteins involved in the countercurrent multiplication process, namely the Na-K-2Cl cotransporter of the thick ascending limb and aquaporin-1, the water channel of the descending limb and proximal tubule.

Materials and Methods

Antibodies

Polyclonal antibodies prepared against aquaporin-1 (12), aquaporin-2 (13), aquaporin-3 (14), aquaporin-4 (15), Na-K-2Cl cotransporter (16), and the type 3 Na-H exchanger (NHE-3) (8) have been described previously. They were raised against keyhole limpet hemocyanin-conjugated synthetic peptides corresponding to hydrophilic...
portions of the amino acid sequences in rat. The specificity of each antibody has been demonstrated by showing unique peptide-antibody bands on immunoblots and a unique distribution of labeling by immunohistochemistry and immunochemistry. These antibodies were affinity-purified against their respective immunizing peptides for use in this study. In addition, a mouse monoclonal antibody against the Na-K-ATPase α1 subunit (Upstate Biotechnology, Lake Placid, NY) was used.

Animal Model
Male Sprague Dawley rats weighing 250 to 300 g were anesthetized with 50 mg of sodium methohexitol, intraperitoneally. Via a midline abdominal incision, the common bile duct was doubly ligated and transected between the two ligatures. The abdominal midline incision was closed in layers, and the rats were allowed to recover from anesthesia and surgery. Immediately after surgery, each rat was placed in an individual metabolic cage and provided with normal rat chow and 5% glucose solution as drinking fluid. We have demonstrated previously that CBDL rats exhibit a continuously positive sodium balance and a normal food and fluid intake in a metabolic balance study over a 26-d period (17). CBDL and control rats were provided with normal rat chow and 5% glucose solution as drinking fluid for the entire 3-wk period.

At the end of 3 wk, urine was collected for 24 h under water-saturated mineral oil. Thereafter, the rats were weighed, anesthetized as above, and, via an abdominal midline incision, arterial blood was collected from the abdominal aorta with a heparinized syringe. The kidneys were removed, individually weighed, inserted in aluminum foil pouches, frozen on dry ice, and stored at −70°C until the day of tissue preparation for immunoblotting. The liver also was weighed. All of the CBDL rats were found to have ascites as evidenced by visible pools of fluid in the lateral abdominal gutters.

All animal experimentation described in this article was conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Blood and Urine Analyses
Serum and urine sodium concentration was measured with a flame photometer (IL943), and serum and urine osmolalities were measured with vapor pressure osmometer (Wescor 5500).

Kidney Dissection and Tissue Preparation for Immunoblotting
The kidneys were washed briefly in ice-cold isolation solution. The left kidneys were dissected to obtain inner medulla, outer medulla, and cortex homogenates. The tissue was initially homogenized for 15 s using a tissue homogenizer (Omni 1000 fitted with a microsawtooth generator) in ice-cold isolation solution containing 250 mM sucrose/10 mM triethanolamine (Calbiochem, La Jolla, CA) with 1 μg/ml leupeptin (Bachem California, Torrance, CA) and 0.1 mg/ml phenylmethylsulfonyl fluoride (United States Biochemical Corp., Toledo, OH). The total protein concentration in each sample was measured using the Pierce BCA Protein Assay reagent kit before addition of Laemmli sample buffer for immunoblotting.

Electrophoresis and Immunoblotting of Membrane Proteins
The membranes were solubilized at 60°C for 15 min in Laemmli sample buffer. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on 8 or 12% polyacrylamide. For each set of samples from vehicle-treated control rats and CBDL-treated rats, an initial gel was stained with Coomassie blue to confirm that equal loading had been achieved as described previously (12). Representative bands were quantified by laser densitometry (Personal Densitometer SI, Molecular Dynamics, San Jose, CA), ensuring that loading did not differ for any sample by more than 10% from the mean. For immunoblotting, the proteins were transferred from unstained gels electrophoretically to nitrocellulose membranes. After being blocked with 5 g/dl nonfat dry milk for 30 min, the blots were probed with the respective antibodies for 24 h at 4°C. After washing, the nitrocellulose membranes were exposed to secondary antibody (donkey anti-rabbit IgG conjugated with horseradish peroxidase, Pierce no. 31458, diluted 1:5000, or rabbit anti-mouse IgG conjugated with horseradish peroxidase, Pierce no. 31434, diluted 1:5000) for 1 h at room temperature. Sites of antibody-antigen reaction were visualized using luminol-based enhanced chemiluminescence substrate (LumiGLO for Western blotting, Kirkegaard and Perry no. VC110). The blots were quantified by densitometry (Molecular Dynamics model PDS1-P90 with ImageQuaNT v4.2 software). The aquaporins typically appear in the immunoblots as two bands corresponding to the nonglycosylated and glycosylated forms of the proteins. When these blots are analyzed by densitometry, both bands are scanned and the densitometry values reported represent the sums of the two bands. The band density values were normalized by dividing by the mean value for the controls and multiplying by 100%.

Statistical Analyses
Quantitative data are presented as mean ± SEM. Statistical comparisons were accomplished by unpaired t test (when variances were the same) or by Mann–Whitney rank sum test (when variances were significantly different between groups). P < 0.05 was considered statistically significant.

Results
Organ Weight, Serum, and Urine Measurements
Table 1 shows results from body weight, serum, and urine measurements for control and CBDL rats. The mean weight of the livers from the CBDL rats was significantly increased relative to controls, whereas kidney weights were not different.
Serum sodium concentration and osmolality were significantly decreased in CBDL rats relative to controls. Despite this, urinary osmolality was substantially greater than plasma osmolality in CBDL rats. Thus, the CBDL rats manifested inappropriate water retention. Urinary sodium excretion was not substantially different between the two groups of rats. Urinary flow rates tended to be lower in the CBDL rats, although there was no significant difference between the two groups.

Expression Levels of Collecting Duct Aquaporins

Aquaporin-2. Figure 1 shows an aquaporin-2 immunoblot using a whole kidney homogenate. As demonstrated previously, immunoblots of aquaporin-2 show two bands (a nonglycosylated band at 29 kD and a glycosylated band at 35 to 40 kD). Aquaporin-2 band density was significantly decreased in this blot (CBDL, 38 ± 11%; control, 100 ± 16%; \( P = 0.01 \)) (see Materials and Methods). Figure 2 shows immunoblots for aquaporin-2 in cortex, outer medulla, and inner medulla. Again, an increase in aquaporin-2 abundance was not found. In fact, there were significant decreases in aquaporin-2 band density in all three regions (Cortex: CBDL, 45 ± 13%; control, 100 ± 34%; \( P = 0.02 \); Outer medulla: CBDL, 25 ± 5%; control, 100 ± 26%; \( P = 0.001 \); Inner medulla: CBDL, 45 ± 32%; control, 100 ± 42%; \( P < 0.0001 \)).

Aquaporin-3. Figure 3 shows aquaporin-3 immunoblots using a whole kidney homogenate and a crude membrane fraction. As was the case for aquaporin-2, we find no evidence for increased aquaporin-3 abundance. Indeed, aquaporin-3 band density was significantly decreased in this blot (CBDL, 9 ± 3%; control, 100 ± 17%; \( P = 0.0003 \)). As shown in the bottom panel of Figure 3, a similar decrease was found in crude membrane fractions from the same kidneys (CBDL, 10 ± 3%; control, 100 ± 20%; \( P = 0.02 \)).

Aquaporin-4. Figure 4 shows an immunoblot for aquaporin-4 in the inner medulla, the only region in which aquaporin-4 is abundant (12). As demonstrated previously, immunoblots of aquaporin-4 show two bands (31 and 52 kD). As for the other two aquaporins, aquaporin-4 expression was significantly decreased (CBDL, 22 ± 7%; control, 100% ± 31%; \( P = 0.003 \)).

Differential Centrifugation: Aquaporin-2

Collecting duct water permeability is regulated acutely by vasopressin by stimulating trafficking of aquaporin-2 vesicles to the plasma membrane. A method for testing for redistribution of aquaporin-2 in collecting duct cells involving differen-

Table 1. Body weight, serum, and urine measurements

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight (g)</th>
<th>LK Weight (g)</th>
<th>Liver Weight (g)</th>
<th>Serum [Na] (mEq/L)</th>
<th>Serum Osm (mosmol/L)</th>
<th>Urinary Flow (ml/24 h)</th>
<th>Urinary [Na] (mEq/24 h)</th>
<th>Urinary Osm (mosmol/L)</th>
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<tr>
<td>Control</td>
<td>376 ± 5</td>
<td>1.23 ± 0.4</td>
<td>11.2 ± 0.5</td>
<td>146 ± 0.6</td>
<td>293 ± 1.8</td>
<td>31.2 ± 2.9</td>
<td>0.68 ± 0.19</td>
<td>1290 ± 112</td>
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<tr>
<td>CBDL</td>
<td>316 ± 19</td>
<td>1.18 ± 0.03</td>
<td>14.0 ± 1.0</td>
<td>134 ± 0.2</td>
<td>270 ± 0.7</td>
<td>22.6 ± 2.1</td>
<td>0.55 ± 0.10</td>
<td>1656 ± 430</td>
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\( a \) Urinary data were measured on final day before sacrifice. Serum samples were obtained at the time of sacrifice. Results are expressed as mean ± SEM. LK, left kidney; Na, sodium; Osm, osmolality; CBDL, common bile duct-ligated rats.

\( b \) \( P < 0.05 \) versus control rats.

Figure 1. Immunoblots comparing abundance of aquaporin-2 in the whole kidney homogenates in kidneys of common bile duct ligation (CBDL) and control rats. Each lane was loaded with a sample from a different rat (whole kidney 2 mg total protein per lane). In this figure, a mature band appears at 35 kD and a nonglycosylated band at 29 kD.

Figure 2. Immunoblots comparing abundance of aquaporin-2 in kidneys of CBDL and control rats. Each lane was loaded with a sample from a different rat (cortex whole homogenate, 10 µg total protein per lane; outer medulla whole homogenate, 3 µg total protein per lane; inner medulla whole homogenate, 1 µg total protein per lane). In all regions, a mature band appears at 35 kD and a nonglycosylated band at 29 kD.

Figure 3. Immunoblots comparing abundance of aquaporin-3 in kidneys of CBDL and control rats. Each lane was loaded with a sample from a different rat (whole kidney homogenate, 10 µg total protein per lane). In this figure, a mature band appears at 35 kD and a nonglycosylated band at 29 kD.
Patial centrifugation was introduced by Marples et al. (18). That study showed that a high density membrane fraction (17,000 \( g \) pellet) contains most of the plasma membranes, whereas a low density membrane fraction (200,000 \( g \) pellet from the 17,000 \( g \) supernatant) is enriched in intracellular vesicles. When redistribution occurs, e.g., in response to vasopressin, there is an increase in the ratio of aquaporin-2 in the high density fraction to aquaporin-2 in the low density fraction (18). Figure 5 shows the results of the application of this technique to the CBDL experiments. As can be seen from Figure 5, aquaporin-2 abundance was proportionately reduced in both membrane fractions (High density membrane fraction: CBDL, 36 \( \pm \) 13%; control, 100 \( \pm \) 25%; \( P = 0.06 \); Low density membrane fraction: CBDL, 47 \( \pm \) 14%; control, 100 \( \pm \) 16%; \( P = 0.03 \)) Thus, there is no evidence from these studies of redistribution of aquaporin-2 within the collecting duct principal cell in CBDL animals.

Expression Level of Aquaporin-1

Figure 6 shows an aquaporin-1 immunoblot using the same whole kidney homogenate as in Figure 1. In contrast to the collecting duct aquaporins, aquaporin-1, which is expressed in proximal tubule and thin descending limb, was not decreased in the CBDL rats (CBDL, 102 \( \pm \) 19%; control, 100 \( \pm \) 16%; \( P = 0.9 \)). An immunoblot using outer medullary homogenates and probed with the aquaporin-1 antibody is shown at the bottom of Figure 6. Although there was a tendency to decrease in the CBDL rats, aquaporin-1 expression level was not significantly different in the two groups (CBDL, 69 \( \pm \) 13%; control, 100 \( \pm \) 18%; \( P = 0.2 \)).

Expression Level of Na-K-2Cl Cotransporter

The Na-K-2Cl cotransporter of the thick ascending limb has a critical role in both urinary concentration and dilution capacity. Figure 7 shows three Na-K-2Cl cotransporter immunoblots using whole kidney homogenates, high density membrane frac-
tions (17,000 × g pellets), and low density membrane fractions (200,000 × g pellets). There was a large decrease in Na-K-2Cl cotransporter band density in all three sets of samples (Whole kidney: CBDL, 10 ± 3%; control, 100 ± 2%; *P* = 0.002; High density membrane fraction: CBDL, 4 ± 5%; control, 100 ± 16%; *P* < 0.0001; Low density membrane fraction: CBDL, 35 ± 6%; control, 100 ± 9%; *P* = 0.0002).

**Expression Levels of NHE-3**

NHE-3 is an important apical sodium transporter in the proximal tubule. Figure 8 shows three NHE-3 immunoblots using whole kidney homogenates, high density membrane fractions (17,000 × g pellets), and low density membrane fractions (200,000 × g pellets). There was a marked decrease in NHE-3 expression in kidneys of CBDL rats that was approximately proportionate in all three sets of samples (Whole kidney: CBDL, 9 ± 4%; control, 100 ± 25%; *P* = 0.004; High density membrane fraction: CBDL, 19 ± 5%; control, 100 ± 19%; *P* = 0.002; Low density membrane fraction: CBDL, 19 ± 5%; control, 100 ± 14%; *P* = 0.002).

**Expression Level of Na-K-ATPase α1 Subunit**

The Na-K-ATPase is the primary active sodium transporter responsible for sodium absorption throughout the nephron. Figure 9 shows three Na-K-ATPase α1 subunit immunoblots using whole kidney homogenates, high density membrane fractions (17,000 × g pellets), and low density membrane fractions (200,000 × g pellets). In contrast to the apical sodium transporters (Na-K-2Cl cotransporter and NHE-3), the expression level of the Na-K-ATPase α1 subunit was not decreased but rather showed a strong tendency to increase (Whole kidney: CBDL, 153 ± 40%; control, 100 ± 25%; *P* = 0.3; High density membrane fraction: CBDL, 276 ± 81%; control, 100 ± 27%; *P* = 0.06; Low density membrane fraction: CBDL, 339 ± 87%; control, 100 ± 25%; *P* = 0.008).

**Discussion**

The experimental model that we investigated in this study was rats that had undergone common bile duct ligation. These animals were studied approximately 3 wk after bile duct ligation. As documented in the study, the animals manifested inappropriate water retention after oral water loading. Specifically, despite the fact that the CBDL rats were hyponatremic, they excreted a urine that was hypertonic relative to plasma (Table 1). We carried out semiquantitative immunoblotting studies aimed at determining whether the CBDL rats manifest specific defects in regulation of the expression of any of the renal aquaporins or Na-K-2Cl cotransporter of the thick ascending limb.

**Aquaporin-2 Abundance Is Decreased in CBDL-Induced Cirrhosis**

Our study confirmed the report of Jonassen *et al.* (11) showing a decrease in aquaporin-2 abundance in CBDL-induced cirrhosis. Previous studies in animal models of extracellular fluid volume (ECF)-expanded states have shown a variety of responses with regard to aquaporin-2 expression. In decompensated congestive heart failure with hyponatremia (4,5), pregnancy (19), and cirrhosis induced by carbon tetrachloride administration (9,10), increases in aquaporin-2 expression were observed. The increases seen in congestive heart failure
are attributable, in part, to increased circulating levels of vasopressin resulting from nonosmotic stimulation of vasopressin release (4). In contrast, rat models of nephrotic syndrome induced by administration of puromycin aminonucleoside (6) and adriamycin (7,8) manifested decreased levels of aquaporin-2 despite high circulating vasopressin levels (6). The results with the CBDL-induced cirrhosis both in the present study and the study of Jonassen et al. (11) are similar to those seen with nephrotic syndrome models, i.e., there was a marked decrease in aquaporin-2 expression. Thus, increased expression of aquaporin-2 is not a uniform finding in rat models of volume-expanded states. Furthermore, in the present study, significant hyponatremia was documented in the CBDL rats in response to water loading by administration of 5% glucose as drinking fluid. Thus, these results show that in the setting of extracellular fluid volume expansion, excessive water retention with hyponatremia can occur in the absence of increases in aquaporin-2 abundance.

In previous studies of hepatic cirrhosis induced by CCl4 administration over a long period (several months), increases in aquaporin-2 expression were observed (9,10) associated with increased levels of circulating vasopressin (20). Thus, findings with these models more nearly resemble the findings in congestive heart failure than those obtained in nephrotic syndrome and the CBDL model of hepatic cirrhosis. This is not surprising in view of the major differences in the pathophysiology of the two types of cirrhosis models. Neither model is necessarily representative of all features of human cirrhosis, and it is possible that human cirrhosis has pathophysiologic features of both models, perhaps manifested at different stages of the disease. Further research, including studies of the molecular pathophysiology of humans with cirrhosis, will be needed to determine how the principles defined in animal studies apply to human disease.

What are the possible explanations for the observed decrease in aquaporin-2 expression in CBDL-induced cirrhosis? Previous studies have demonstrated that an important factor in the regulation of aquaporin-2 expression is the circulating level of vasopressin (13). Although we did not measure circulating vasopressin levels in this study, a similar study of the long-term effect of the CBDL in rats did not show a decline in circulating vasopressin levels, but did show a decrease in aquaporin-2 expression (11). Studies of the vasopressin escape phenomenon have demonstrated that factors other than vasopressin can regulate aquaporin-2 expression (21). In particular, water loading in the presence of high levels of antidiuretic hormone was associated with a selective decrease in aquaporin-2 expression without effects on aquaporin-3 and aquaporin-4. As demonstrated in the present study, CBDL-induced cirrhosis is associated with decreases in all three collecting duct aquaporins, suggesting that the decrease in aquaporin-2 expression is not the result of the vasopressin escape phenomenon. It is also possible that toxic substances that accumulate in the blood as a result of biliary ligation can affect renal function. Conceivably, such substances could have a direct effect on the collecting duct to suppress aquaporin expression. The downregulation of aquaporin-2 expression seen with lithium toxicity (22), hypokalemia (23), and ureteral ligation (24) also remain unexplained and could involve a mechanism similar to that responsible for decreased expression of aquaporin-2 in CBDL-induced cirrhosis. An additional possible explanation for decreased aquaporin-2 expression in both nephrotic syndrome and CBDL-induced cirrhosis is that chronic decreases in tubule fluid delivery may result in a suppression of collecting duct transporter expression. Micropuncture studies in CBDL-induced cirrhosis in dogs (25) demonstrated an increase in proximal fluid reabsorption. Similar findings had been obtained in rats with cirrhosis secondary to carbon tetrachloride administration (26). An increase in proximal tubule fluid reabsorption would necessarily reduce distal delivery. It has been well established that chronic increases in tubule fluid delivery to the distal nephron in a setting of chronic furosemide administration (27) or reduced renal mass (28) result in collecting duct hypertrophy. The hypertrophy with chronic furosemide administration is associated with an increase in aquaporin-2 expression (29). Whether a chronic decrease in tubule fluid flow rate (resulting from increased proximal reabsorption) directly decreases aquaporin-2 expression and the expression of the other collecting duct aquaporins remains to be investigated. Measurements of distal delivery of fluid were not carried out in the present study. The relatively low urinary flow rates seen in the CBDL animals (22.6 ml/24 h) despite extremely low levels of collecting duct aquaporins suggest that flow rates entering the collecting ducts were indeed low.

**Water Retention**

In the present study, the CBDL rats developed significant hyponatremia and decreased serum osmolality in response to stimulation of drinking behavior by giving sweetened water. The hyponatremia signifies that the CBDL rats inappropriately
retained water. Furthermore, the urine was concentrated above plasma osmolality despite systemic hypo-osmolality. How can the water retention be explained? Based on the findings in this study, it is unlikely that collecting duct water permeability was increased. Indeed, the profound decrease in expression levels of all three collecting duct aquaporins suggests that collecting duct water permeability was likely to have been decreased. Previous studies have demonstrated that urine can be concentrated to osmolalities higher than plasma osmolality even in the absence of circulating vasopressin (30,31). Based on studies involving renal arterial clamping in dogs with central diabetes insipidus, it was inferred that this vasopressin-independent concentration process was dependent on reduced flow in the distal nephron and collecting ducts (31). The explanation for this phenomenon is that collecting duct water permeability does not fall to zero even in the absence of vasopressin (32,33). Consequently, if the luminal flow rate is slow enough, osmotic equilibration across the collecting duct epithelium can still occur. As noted in the previous paragraph, there is evidence for a strong increase in proximal tubule fluid reabsorption in CBDL-induced cirrhosis (25). Thus, it can be speculated that distal delivery is indeed diminished in CBDL-induced cirrhosis, resulting in partial osmotic equilibration despite decreased aquaporin expression. Other factors that could have contributed to inappropriate water retention include inappropriately high water permeability in the cortical thick ascending limb or distal convoluted tubule or a markedly enhanced countercurrent multiplier mechanism. This study provides no direct evidence regarding these possibilities. However, it appears unlikely that there is a marked increase in countercurrent multiplication in the kidneys of CBDL rats because the expression level of the thick ascending limb Na-K-2Cl cotransporter was markedly suppressed. This is consistent with the decreased concentration capacity observed in cirrhosis (34).

**Downregulation of Aquaporin-3 and Aquaporin-4**

Most studies of the molecular basis of collecting duct water transport abnormalities in ECF volume-expanded states have focused on the apical water channel, aquaporin-2. However, it must be remembered that water transport across the cells of the collecting duct depends on water transport across both the apical and basolateral plasma membranes. Thus, the basolateral water channels aquaporin-3 and aquaporin-4 could play important roles in abnormalities of collecting duct water transport. This point was highlighted by a recent study in which aquaporin-4 gene was “knocked out” in mice (35). The inner medullary collecting ducts exhibited a fourfold reduction of osmotic water permeability despite normal expression of aquaporin-2. In the current studies, marked reductions in both aquaporin-3 and aquaporin-4 expression were observed, predicting a marked impairment of collecting duct water permeability independent of any effects due to altered aquaporin-2 regulation. Similar decreases in aquaporin-3 and -4 expression were seen in nephrotic syndrome models (6,7). Additional studies are required to assess the role of altered regulation of aquaporin-3 and -4 in other ECF volume-expanded states.

**Downregulation of Na-K-2Cl Cotransporter**

The CBDL model shares another characteristic with nephrotic syndrome in rats (8), i.e., a marked decrease in Na-K-2Cl cotransporter expression. The mechanism of the downregulation of Na-K-2Cl cotransporter expression has not been addressed in this study. However, several recent studies provide a basis for speculation. Both vasopressin (16) and saline loading (29) increase Na-K-2Cl cotransporter expression. As discussed above, vasopressin levels are unlikely to be decreased in this model. The mechanism of the saline loading effect has not been established, but it is likely to be related to ECF volume expansion. In addition, recent studies have demonstrated that prostaglandin E₂ (PGE₂), working through the EP3 receptor, decreases Na-K-2Cl cotransporter expression in the thick ascending limb (36). Whether there are increased levels of interstitial PGE₂ surrounding the thick ascending limb in CBDL-induced cirrhosis is unknown. In general, many studies on the potential role of PGE₂ and other prostanoids have provided strong support for the view that disordered salt and water excretion in cirrhosis is, at least in part, dependent on the action of cyclo-oxygenase products (37). It is interesting that another recent study reporting Na-K-2Cl cotransporter expression levels in CBDL rats did not demonstrate a fall in Na-K-2Cl cotransporter expression (11). This raises the possibility that the decrease in Na-K-2Cl cotransporter expression seen in the present study was dependent on the administration of 5% glucose drinking fluid, which was not done in the previous study. The CBDL rats in the present study had visible ascites in contrast to what was reported by Jonassen et al. (11). Thus, there is evidence of greater extracellular volume expansion in our study, which may play a role in suppression of Na-K-2Cl cotransporter expression.

**Na-K-ATPase**

Although expression levels of both the Na-K-2Cl cotransporter and NHE-3 (a major apical sodium transporter in the proximal tubule) were decreased, it was interesting that the expression level of the Na-K-ATPase was not decreased, but instead showed a propensity to increase. This observation is interesting for two reasons. First, it showed that decreased transporter expression was not due to a global decrease in expression of all plasma membrane proteins along the nephron. Thus, the mechanism of suppression of gene expression in the CBDL model is selective. Second, the maintenance of normal to high levels of Na-K-ATPase expression, despite decreased tubule fluid flow rates (25,26), may point to a role for altered Na-K-ATPase regulation in the sodium retention that accompanies cirrhosis. Additional studies will be needed to test this hypothesis.

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