Effects of renal denervation on tubular sodium handling in rats with CBL-induced liver cirrhosis

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1Department of Pharmacology, University of Copenhagen, DK-2200 Copenhagen N; 2The Water and Salt Research Center, Institute of Anatomy, and 4University Institute of Pathology, Aarhus Kommune Hospital, University of Aarhus, DK-8000 Aarhus C; and 3Department of Physiology and Pharmacology, University of Southern Denmark, DK-5000 Odense C, Denmark
Submitted 18 July 2002; accepted in final form 6 November 2002

Jonassen, Thomas E. N., Lone Brønd, Malene Torp, Martin Græbe, Søren Nielsen, Ole Skøtt, Niels Marcussen, and Sten Christensen. Effects of renal denervation on tubular sodium handling in rats with CBL-induced liver cirrhosis. Am J Physiol Renal Physiol 284: F555–F563, 2003.—This study was designed to examine the effect of bilateral renal denervation (DNX) on thick ascending limb of Henle's loop (TAL) function in rats with liver cirrhosis induced by common bile duct ligation (CBL). The CBL rats had, as previously shown, sodium retention associated with hypertrophy of the inner stripe of the outer medulla (ISOM) and increased natriuretic effect of furosemide in vivo, and semi-quantitative immunoblotting showed increased expression of the furosemide-sensitive Na-K-2Cl cotransporter type 2 (NKCC2) in ISOM from CBL rats. DNX significantly attenuated the sodium retention in the CBL rats, which was associated with normalization of the natriuretic effect of furosemide, as well as a significant reduction in the expression of NKCC2 in the ISOM. However, the marked hypertrophy of the ISOM found in CBL rats was not reversed by DNX. Together, these data indicate that increased renal sympathetic nerve activity known to be present in CBL rats plays a significant role in the formation of sodium retention by stimulating sodium reabsorption in the TAL via increased renal abundance of NKCC2.

common bile duct ligation; furosemide-sensitive sodium-potassium-2chloride cotransporter type 2; thick ascending limb; furosemide; sodium balance; inner stripe of outer medulla

It is well described that rats with liver cirrhosis induced by common bile duct ligation (CBL) develop edema and ascites. Intact renal innervation seems to play a key role in homeostatic regulatory responses to sodium depletion and sodium loading, and it has been suggested that ~30–40% of the renal sodium retention during edema-forming conditions such as liver cirrhosis (12), congestive heart failure (12), and nephrotic syndrome (18) is dependent on intact renal sympathetic innervation. Autoradiographic studies have shown intense norepinephrine labeling throughout the renal tubules (4–7), and renal sympathetic nerve (RSN) stimulation, which does not affect renal blood flow or glomerular filtration rate (GFR), causes a reversible decrease in urinary sodium excretion in rats (39). Moreover, free-flow micropuncture and tubular microperfusion studies have shown that this stimulatory effect of RSN on tubular sodium reabsorption occurs throughout the tubule (11) and the magnitude of the stimulatory effect of RSN seems to be proportional to the density of the renal tubular innervation, being greatest in the thick ascending limb of Henle's loop (TAL) and least in the collecting duct (4). Together, these findings indicate that RSNs directly stimulate renal tubular sodium reabsorption.

We recently investigated renal function in rats with CBL-induced liver cirrhosis (20, 22, 24) and found that the rats had increased natriuretic response to furosemide together with marked hypertrophy of the TAL epithelium in the inner stripe of the outer medulla (ISOM). Moreover, the capacity to increase the medul- lary interstitial sodium concentration in response to thirsting was enhanced in the CBL rats (23). Together, these observations indicate that increased sodium reabsorption in the TAL plays a significant role in the sodium retention, which eventually will result in the formation of edema and ascites. Interestingly, recent studies from our own as well as other laboratories show that the furosemide-sensitive Na-K-2Cl cotransporter type 2 (NKCC2) exclusively expressed in the TAL and macula densa is significantly increased in other conditions with sodium retention, such as congestive heart failure and sepsis (21, 32, 35).

The present study was therefore designed to examine the effect of bilateral renal denervation on sodium retention as well as renal function and structure, including the expression of NKCC2 and other TAL transporters, the luminal electroneutral sodium-proton exchanger (NHE3) (9), and the basolateral Na-K-ATPase in rats with CBL-induced liver cirrhosis. Renal function was examined in chronically instrumented rats...
during control conditions and during acute administration of furosemide. To prevent furosemide-induced sodium and water depletion, we used a computerized servo-controlled sodium- and water-replacement system, where losses of sodium and water were replaced momentarily (19).

METHODS

Experimental Animals

Female Wistar rats (230–250 g) from Charles River (Sulzfeld, Germany) were used for the experiments. The animals were housed in a temperature (22°C ± 1°C) and humidity (40–70%)-controlled room with a 12:12-h light-dark cycle (light on from 6:00 AM to 6:00 PM). Animals were given free access to tap water and a diet containing ~140 mmol/kg of sodium, ~275 mmol/kg of potassium, and 23% protein. All animal procedures followed the guidelines for the care and handling of laboratory animals established by the Danish government.

Animal Preparation

Cirrhosis was induced by CBL as described by Kountouras and co-workers (28). Briefly, biliary obstruction induces portal inflammation and bile duct proliferation, which eventually will result in the formation of cirrhosis. Control rats were subjected to sham-CBL.

Bilateral renal denervation (DNX) was performed through flank incisions. The adventitia of the renal vein and artery were carefully dissected under a microscope. All visible nerves were cut, and the vessels were coated with 10% phenol in 95% ethanol. With this procedure, renal norepinephrine content is reduced to <5% of control levels (37).

Three weeks after CBL/sham-CBL and renal denervation/sham denervation, permanent medical-grade Tygon catheters were implanted in the femoral artery and vein and a permanent suprapubic bladder catheter was implanted in the bladder as described previously (22, 38). After instrumentation, the animals were housed individually.

Experimental Groups

The experimental groups were as follows: sham (sham-CBL rats with sham-DNX); sham-DNX (sham-CBL rats with bilateral DNX); CBL (CBL rats with sham-DNX); and CBL-DNX (CBL rats with bilateral DNX).

The experiments were performed in animals in two series: series 1 (n = 6–8/group), in which sodium balance studies were performed, plasma samples for measurement of renin and aldosterone were collected, and kidneys were perfused fixed; and series 2 (n = 7–8/group), in which renal functions studies were performed, and kidneys were used for immunoblotting.

Series 1

Sodium balance studies. Four weeks after CBL or sham operation, the rats were placed in metabolic cages for accurate determination of daily food and water intake. After 2 days of adaptation, sodium balance was measured daily for 3 consecutive days, and the average of the three values was used. Sodium intake was calculated from the amount of diet ingested per 24 h, and sodium loss was estimated from the amount of sodium excreted in the urine within the same 24 h. Sodium balance was then calculated as the difference between sodium intake and sodium excretion.

Measurement of plasma renin and aldosterone. Five weeks after CBL or sham operation (i.e., 2 days after the termination of the sodium balance studies), the rats were placed in restraining cages, arterial blood samples (total volume 1.0 ml) were drawn from a permanent arterial catheter, and the plasma was stored at −20°C for later measurement of renin and aldosterone. Plasma renin concentration was measured by ultramicroradioimmunoassay of generated ANG I with the “antibody-trapping” technique of Lykkegaard and Poulsen (30). Aliquots of plasma were diluted 20- to 80-fold with Tris buffer containing human albumin, and 5-μl portions of these samples were incubated for 24 h at 37°C with 20 μl of a reaction mixture that contained purified rat renin substrate (~1,200 ng ANG I equivalents/ml). This incubation was followed by radioimmunoassay of generated ANG I.

Plasma aldosterone concentration was measured in reference to renin standards obtained from the National Institute for Biological Standards and Control (Potters Bar, Herts, UK; 1 milli-Goldblatt unit = 160 pg ANG I·ml⁻¹·h⁻¹). Plasma aldosterone concentration was measured by radioimmunoassay using a commercial kit (Coat-A-Count Aldosterone, DPC, Los Angeles, CA).

Histological examinations. Then, the rats were anesthetized with halothane-nitrous oxide, and the left kidney was perfused in vivo for 3 min with 1.5% glutaraldehyde in Tyrode’s solution with added 2.25% dextran T-40 (perfusion pressure: 150 mmHg) and postfixed in perfusion fluid for later stereological examination. The kidneys were sliced at a 90° angle on the longitudinal axis of the kidney. The 2-mm-thick slices were embedded in paraffin, and 3- to 4-μm-thick sections were cut and stained with hematoxylin-eosin. From this, the volume fractions of the different renal zones were measured stereologically (17). All sections were investigated by light microscopy point counting (using a stage motor), in systematic order with random starting points. The number of points hitting within each zone was estimated. The total number of hitting points within each kidney slice was 200–300, and each field of vision included a grid with 4 points. Data from all 2-mm-thick slices were included, which means that approximately five to seven sections from each kidney were examined. When the volume fractions of the different zones are known, the absolute volumes of the zones can be calculated by multiplying the volume fractions with the volume of the kidney (equal to the kidney weight, assuming that the specific gravity of the kidney is 1 g/cm³) (22).

Series 2

Renal clearance studies. Renal hemodynamic and tubular responses to furosemide were examined by clearance techniques in conscious, chronically instrumented rats 4 wk after a CBL/sham-CBL operation. Before the clearance experiments, all rats were adapted to the restraining cage used for these experiments by training them for two periods of 2 h each on consecutive days. Clearance experiments were started at 8:00 AM. Clearance of [³⁵S]taetraethylammonium bromide was used as a marker for effective renal plasma flow, clearance of [³H]inulin as a marker for GFR, and clearance of lithium (CLi) as a marker for distal delivery (42). In addition to minor amounts of lithium in the infusion solutions, lithium was added to the diet (12 mmol/kg diet) for 3 days before the experiments to avoid acute effects of lithium on renal function (29). The clearance experiments were performed as follows. Clearance markers in 150 mM glucose, 13 mM NaCl, and 3 mM LiCl were infused at a constant rate of 2.5 ml/h.
Table 1. Twenty-four-hour urine production, sodium intake, sodium excretion, and sodium balance in rats 5 wk after liver cirrhosis was induced by common bile duct ligation

<table>
<thead>
<tr>
<th></th>
<th>Sham (n = 7)</th>
<th>Sham-DNX (n = 8)</th>
<th>CBL (n = 10)</th>
<th>CBL-DNX (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24-h Urine production, ml-day⁻¹·100 g BW⁻¹</td>
<td>9.1 ± 1.0</td>
<td>9.4 ± 1.3</td>
<td>8.5 ± 2.3</td>
<td>14.3 ± 3.4†</td>
</tr>
<tr>
<td>24-h Sodium intake, μmol Na ingested/day</td>
<td>1,248 ± 137</td>
<td>1,201 ± 104</td>
<td>1,299 ± 88</td>
<td>934 ± 168†</td>
</tr>
<tr>
<td>24-h Sodium excretion, μmol Na excreted/day</td>
<td>1,489 ± 129</td>
<td>1,502 ± 147</td>
<td>1,533 ± 162</td>
<td>1,437 ± 154</td>
</tr>
<tr>
<td>24-h Sodium balance, μmol Na retained/day</td>
<td>1,380 ± 158</td>
<td>1,305 ± 181</td>
<td>1,084 ± 121†</td>
<td>1,221 ± 98</td>
</tr>
<tr>
<td>Body wt, g</td>
<td>109 ± 116</td>
<td>197 ± 124</td>
<td>449 ± 60†</td>
<td>215 ± 102†</td>
</tr>
</tbody>
</table>

Values are means ± SE of the average of collections on 3 consecutive days. n, No. of rats; DNX, bilateral renal denervation; CBL, common bile duct ligation; BW, body wt. Sodium balance was calculated as sodium intake − sodium excretion. Sham-CBL rats were used as controls. Rats either underwent DNX or were sham denervated at the time of CBL/sham-CBL. †P < 0.05 vs. sham. *P < 0.05 vs. CBL.

Throughout the experiments, after a 90-min equilibration period where steady-state levels of the tracer substances were reached, urine was collected in 2 × 30-min control periods to characterize baseline values of systemic and renal hemodynamics and tubular function. Then, infusion of furosemide was started at a constant rate of 0.50 mg/h, and urine was collected in 8 × 30-min periods during furosemide infusion. To avoid sodium and water depletion, all furosemide-induced water and sodium losses were immediately replaced using a computerized servo-controlled water- and sodium-replacement system (19, 28), which originally was developed by Andersen and Bie (2) for sodium and water replacement in dogs. The servo system consists of a sodium-sensitive electrode (Radiometer) that continuously measures sodium concentration in the urine, a balance (Sartorius model MC 1, Göttingen, Germany) that registers urine production (integration period: 1 min), and two infusion pumps (model 200–2250 CA liquid scintillation counter. Renal clearances (C) and fractional excretions (FE) were calculated by the standard formula

\[ C = U \times V / P; \quad FE = C / GFR \]

where U is concentration in urine, V is urine flow rate, and P is plasma concentration.

Semiquantitative immunoblotting. Five days after the renal clearance studies, the rats were anesthetized with halothane/N₂O, and the right kidney was removed, immediately frozen in liquid nitrogen, and stored at −80°C until processing for membrane fractionation. The outer medulla was isolated and homogenized using a tissue homogenizer (Ultra-Turrax T25, Ika, Staufen, Germany) in 10 ml of ice-cold homogenizing buffer containing 300 mM sucrose, 25 mM HEPES-Tris (pH 7.4), 250 mM NaCl, and 5% (v/v) glycerol. After 60-min blocking in 5% milk in PBS-T buffer, membranes were probed overnight at 4°C with the appropriate primary antibody. For measurement of NKCC2, we used a rabbit polyclonal anti-NKCC2 antibody raised to a synthetic peptide corresponding to amino acids 33–55 of rat NKCC2.

Table 2. Body weight and plasma levels of renin and aldosterone in rats 5 wk after liver cirrhosis was induced by common bile duct ligation

<table>
<thead>
<tr>
<th></th>
<th>Sham (n = 7)</th>
<th>Sham-DNX (n = 8)</th>
<th>CBL (n = 8)</th>
<th>CBL-DNX (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>258 ± 7</td>
<td>249 ± 7</td>
<td>241 ± 6</td>
<td>251 ± 8</td>
</tr>
<tr>
<td>Plasma renin, 10⁻⁸ GU/ml</td>
<td>21.7 ± 4.8</td>
<td>23.8 ± 4.1</td>
<td>25.6 ± 3.6</td>
<td>22.0 ± 4.5</td>
</tr>
<tr>
<td>Plasma aldosterone, nM</td>
<td>1.8 ± 0.6</td>
<td>1.5 ± 0.5</td>
<td>2.3 ± 0.7</td>
<td>1.9 ± 0.4</td>
</tr>
<tr>
<td>Plasma Na, mM</td>
<td>139.1 ± 1.1</td>
<td>139.5 ± 1.1</td>
<td>140.7 ± 1.1</td>
<td>141.0 ± 0.9</td>
</tr>
<tr>
<td>Plasma K, mM</td>
<td>4.5 ± 0.1</td>
<td>4.2 ± 0.1</td>
<td>4.5 ± 0.2</td>
<td>4.3 ± 0.1</td>
</tr>
<tr>
<td>Plasma osmolality, mosmol/kgH₂O</td>
<td>291 ± 2</td>
<td>293 ± 2</td>
<td>292 ± 1</td>
<td>291 ± 2</td>
</tr>
</tbody>
</table>

Values are means ± SE, n, No. of rats. Sham-CBL rats were used as controls. Rats either underwent DNX or were sham denervated at time of CBL/sham-CBL.

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Table 3. Effects of bilateral renal denervation on mean arterial pressure, effective renal plasma flow, glomerular filtration rate, effective filtration fraction, lithium clearance, and fractional lithium excretion in rats 5 wk after liver cirrhosis was induced by common bile duct ligation.

<table>
<thead>
<tr>
<th></th>
<th>Sham (n = 8)</th>
<th>Sham-DNX (n = 8)</th>
<th>CBL (n = 8)</th>
<th>CBL-DNX (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP, mmHg</td>
<td>112 ± 5</td>
<td>107 ± 6</td>
<td>104 ± 3</td>
<td>107 ± 5</td>
</tr>
<tr>
<td>ERPF, μl/min 100 g BW⁻¹</td>
<td>4.395 ± 341</td>
<td>4.338 ± 278</td>
<td>4.308 ± 442</td>
<td>4.370 ± 300</td>
</tr>
<tr>
<td>GFR, μl/min 100 g BW⁻¹</td>
<td>1.938 ± 87</td>
<td>1.131 ± 51</td>
<td>789 ± 61*</td>
<td>926 ± 69</td>
</tr>
<tr>
<td>EFF, %</td>
<td>25.0 ± 0.6</td>
<td>26.5 ± 1.2</td>
<td>18.8 ± 0.8a</td>
<td>21.5 ± 1.6a</td>
</tr>
<tr>
<td>CLi, μl/min 100 g BW⁻¹</td>
<td>239 ± 29</td>
<td>336 ± 51*</td>
<td>197 ± 30</td>
<td>290 ± 20*</td>
</tr>
<tr>
<td>FEli, %</td>
<td>22.1 ± 2.7</td>
<td>28.7 ± 1.3*</td>
<td>23.8 ± 2.9</td>
<td>28.6 ± 2.2</td>
</tr>
</tbody>
</table>

Values are means ± SE. n, No. of rats; MAP, mean arterial pressure; ERPF, effective renal plasma flow; GFR, glomerular filtration rate; EFF, effective filtration fraction; CLi, lithium clearance; FEli, fractional lithium excretion. Sham-CBL rats were used as controls. Rats either underwent DNX or were sham denervated at the time of CBL/sham-CBL. *P < 0.05 vs. sham. †P < 0.05 vs. CBL.

(14). For measurement of NHE3, we used a rabbit polyclonal anti-NHE3 antibody raised against a synthetic peptide corresponding to amino acids 809–831 of rat NHE3 (36). For measurement of Na-K-ATPase, we used a commercial rabbit polyclonal anti-α1-subunit antibody (06–520; Upstate Biotechnology, Lake Placid, NY). Finally, for measurement of aquaporin-2 (AQP2), we used a commercial affinity-purified goat polyclonal antibody (sc-9882; Santa Cruz Biotechnology, Santa Cruz, CA).

The labeling was visualized with horseradish peroxidase-conjugated secondary antibody [P448 (rabbit) or P0449 (goat); Dako; diluted 1:3,000] using an enhanced chemiluminescence system (ECL+, Amersham). The broad ~165-kDa band corresponding to NKCC2, the ~86-kDa band corresponding to NHE3, the ~96-kDa band corresponding to Na-K-ATPase or the 29- and 35- to 50-kDa bands corresponding to AQP2 and glucosylated AQP2, respectively, were scanned by use of a FluorX multi-imager (Bio-Rad Laboratories). Densitometry of individual bands was quantitated using the software program Quantity One, version 4.2.3 (Bio-Rad Laboratories). Protein labeling in samples from the different groups was expressed relative to the mean expression of the control material run on the same gel.

Statistics

Data are presented as means ± SE. To evaluate the effect of furosemide, the average values during the two 30-min control periods were compared with the average values during the last three 30-min periods of the furosemide-induced diuresis. Comparisons were performed by two-way analysis of variance followed by Fisher’s least significant difference test. Differences were considered significant at the 0.05 level.

RESULTS

Daily sodium intake was similar in all groups, but daily sodium excretion significantly decreased in the CBL rats, which indicated the presence of sodium retention relative to the sham-operated control animals (Table 1). DNX had no effect on sodium handling in the sham-operated control rats, but sodium retention was significantly attenuated in CBL-DNX rats (Table 2).

Twenty-four-hour urine production and urine osmolality were similar in the sham and CBL rats. DNX had no effect on urine production or urine osmolality in the sham-operated rats, but in CBL rats DNX significantly increased the production of solute-free urine. Plasma levels of renin, aldosterone, sodium, and potassium as well as plasma osmolality were similar in all four groups (Table 2).

Renal Function Studies

Baseline values. MAP and effective renal plasma flow (ERPF) were similar in all four groups (Table 3). GFR was decreased compared with controls, and the effective filtration fraction was therefore, as previously shown (22, 23, 25), significantly decreased in the CBL rats. DNX had no significant effects on effective filtration fraction or GFR in either CBL or sham-CBL rats. There were no significant differences in CLi between the CBL and sham-CBL groups, whereas DNX increased CLi in both the CBL and the sham-CBL rats. Baseline values for V and urinary sodium excretion rate (UNaV) were similar in all groups (data not shown).

Effect of furosemide on renal sodium handling. Constant furosemide infusion under conditions in which water and sodium depletion was prevented by use of a computerized servo-controlled system induced a prolonged and sustained diuretic and natriuretic response in all four groups. Within all the groups, diuresis and natriuresis reached a steady state after an ~150-min infusion (Fig. 1). The furosemide-induced natriuresis was as previously shown (20, 22–24) to be significantly increased in the CBL rats [ΔUNaV (CBL: 22.9 ± 3.1 vs. sham: 13.4 ± 2.0 μmol·min⁻¹·100 g body wt⁻¹, P < 0.05); ΔFEli (CBL: 20.2 ± 2.6 vs. sham: 10.2 ± 1.7%, P < 0.01)]. Similarly, the change in fractional distal sodium excretion (CNa/Cli) was significantly increased in the CBL rats [ΔCNa/Cli (CBL: 30.8 ± 3.9 vs. sham: 19.8 ± 2.4%, P < 0.01)]. DNX normalized the natriuretic response to furosemide. The furosemide-induced increase in fractional sodium excretion was as previously shown (20, 22–24) to be significantly increased in the CBL rats [ΔCNa/Cli (CBL: 30.8 ± 3.9 vs. sham: 19.8 ± 2.4%, P < 0.01)]. DNX normalized the natriuretic response to furosemide.

1Renal clearance experiments were performed during the inactive period of the rat (i.e., during the daytime), when sodium- and water-retaining mechanisms are maximally activated. To get stable urine production under these conditions, all rats were slightly water-loaded by infusion of a hypotonic glucose solution (2.5 ml/h). Therefore, as previously described, baseline levels of urine flow rate (V) were similar (i.e., clamped) in all four groups as previously demonstrated (23, 25). Furthermore, in accordance with previous studies performed during the daytime and with a low sodium infusion rate (infusion rate: 32.5 μmol Na/h) (23–25), renal sodium handling was similar in all four groups.
uretic response to furosemide in CBL rats [ΔUNaV (CBL-DNX: 14.6 ± 2.1 vs. CBL: 22.9 ± 3.1 μmol·min⁻¹·100 g body wt⁻¹, P < 0.05); ΔFENa (CBL-DNX: 11.7 ± 2.6 vs. CBL: 20.2 ± 2.6%, P < 0.01); ΔCNa/Cli (CBL-DNX: 19.3 ± 2.9 vs CBL: 30.8 ± 3.9%, P < 0.01)]. DNX had no significant effect on the furosemide-induced natriuretic response in the sham-CBL rats. Furosemide produced similar changes in MAP, GFR, ERPF, and FELi in all four groups (data not shown).

Renal histopathology. The stereological analysis performed on the in vivo perfusion-fixed kidneys showed, in accordance with previous reports from our laboratory (20, 22), that CBL rats had marked and selective hyperthrophy of the ISOM (absolute volume: 154 ± 17 vs. 227 ± 14 mm³, P < 0.01). DNX had no effect on the hypertrophy of the ISOM in CBL rats (Fig. 2).

Expression of sodium transporters in renal outer medulla. Figure 3A shows an immunoblot of membrane fractions (40 μg protein/lane) from renal outer medullary preparations. The affinity-purified anti-NKCC2 antibody recognizes a broad band at ~165 kDa corresponding to the furosemide-sensitive type-2 Na-K-2Cl cotransporter exclusively expressed in the TAL and in the macula densa (14). Densitometry of all samples (Fig. 3B) revealed an increased expression of NKCC2 in the outer medulla in CBL rats compared with sham-operated controls (sham: 100 ± 14 vs. CBL: 131 ± 4%, P < 0.05). DNX significantly decreased the expression of NKCC2 in CBL rats, as shown in Fig. 3C and E. Results of the densitometry were CBL: 100 ± 15 vs. CBL-DNX: 57 ± 11%, P < 0.05, and sham: 100 ± 27 vs. CBL-DNX: 62 ± 21%, not significant. DNX had no effect on NKCC2 expression in the sham-DNX rats.
Figure 3. Immunoblots of membrane fractions (40 μg protein/lane) from a renal outer medullary preparation. The rats were subjected to CBL or sham operation (sham) 5 wk earlier and either underwent DNX or were sham denervated at the time of CBL/sham-CBL. The immunoblots were reacted with affinity-purified anti-Na⁺-K⁺-2Cl⁻ cotransporter (NKCC2) and reveal a band at ~165 kDa. A and B: immunoblot showing sham vs. CBL and densitometry performed on all samples, respectively. C and D: immunoblot showing CBL vs. CBL-DNX and densitometry performed on all samples, respectively. E and F: immunoblot showing sham vs. CBL-DNX and densitometry performed on all samples, respectively. Values are means ± SE. *P < 0.05 vs. sham. #P < 0.05 vs. CBL.

Figure 4. Immunoblots of membrane fractions (40 μg protein/lane) from a renal outer medullary preparation. The rats were subjected to CBL or sham operation (sham) 5 wk earlier and either underwent DNX or were sham denervated at the time of CBL/sham-CBL. The immunoblots were reacted with affinity-purified anti-Na⁺-H⁺ cotransporter (NHE3) and reveal a band at ~86 kDa. A and B: immunoblot showing sham vs. CBL and densitometry performed on all samples, respectively. C and D: immunoblot showing CBL vs. CBL-DNX and densitometry performed on all samples, respectively. E and F: immunoblot showing sham vs. CBL-DNX and densitometry performed on all samples, respectively. Values are means ± SE. *P < 0.05 vs. sham.
proximal tubules (9). Densitometry of all samples (Fig. 4B) revealed a decreased expression of NHE3 in the outer medulla of CBL rats compared with sham-operated controls (sham: 100 ± 8 vs. CBL: 57 ± 4%, P < 0.05). Renal denervation had, as shown in Fig. 4, C–F, no effect on the expression of NHE3 in CBL rats. Similarly, DNX had no effect on NHE3 expression in the sham-DNX rats (data not shown).

We also measured the abundance of the α1-subunit of the Na-K-ATPase in the outer medulla. Na-K-ATPase is expressed in both the TAL and the collecting ducts. All our measurements showed that the abundance of the α1-subunit was similar in sham and CBL rats and that DNX had no effect on the expression level in either sham or CBL rats (data not shown).

**Expression of AQP2 in the renal outer medulla.** Finally, we measured the expression level of AQP2 protein in the outer medulla. AQP2 is expressed in the principal cells of the collecting ducts, and the affinity-purified anti-AQP2 antibody recognizes the 29-kDa and the 35- to 50-kDa band, corresponding to nonglycosylated and glycosylated AQP2 protein, respectively. In accordance with previous reports from our laboratory (23, 25), AQP2 expression was significantly decreased in CBL rats (Fig. 5, A and B). As shown in Fig. 5, C–F, renal denervation had no effect on the expression of AQP2 in CBL rats. Similarly, the expression of AQP2 was unchanged in the sham-DNX rats (data not shown).

**DISCUSSION**

The major finding of the present study is that DNX ameliorates the development of renal tubular dysfunction in rats with CBL-induced liver cirrhosis by mechanisms most probably involving inhibition of increased sodium reabsorption in the TAL. The sodium balance studies revealed that DNX normalized 24-h sodium balance, and the clearance studies showed that DNX normalized the increased natriuretic response to furosemide found in CBL rats. Moreover, DNX significantly reduced the expression of NKCC2. However, the marked hypertrophy of the ISOM found in CBL rats was not reversed by DNX.

An increasing number of studies have evaluated TAL function in conditions with impaired renal sodium handling. The present study confirmed previous findings from our own laboratory (20, 22–24, 26) indicating that sodium reabsorption in the TAL is increased in cirrhotic rats and plays a significant role in the sodium retention that eventually results in the formation of edema and ascites. However, not only liver cirrhosis seems to be associated with altered TAL function. Increased NKCC2 expression has also been found in rats with congestive heart failure (32, 35, 41), and recently we have shown that sepsis-induced acute renal failure is associated with increased NKCC2 expression (26). Moreover, Alvarez-Guerra and Garay (1) have reported increased natriuretic effect of bumetanide associated with increased bumetanide-sensitive rubidium uptake in TAL from Dahl-S hypertensive rats (9).
rats, and Manning and co-workers (31) have recently shown increased NKCC2 expression in rats with prenatally programmed hypertension induced by a maternal low-protein diet during pregnancy. Together, these data seem to support a role of regulation of NKCC2 abundance in a number of pathophysiological conditions with impaired renal sodium handling.

RSNs are important modulators of renal sodium excretion through release of the neurotransmitter noradrenaline. DiBona and co-workers (13) have demonstrated that RSN activity is increased during conditions with extracellular volume expansion, including liver cirrhosis and congestive heart failure (13). Moreover, long-term sodium balance studies have shown that renal denervation significantly attenuates the development of excess sodium accumulation in rats with liver cirrhosis or congestive heart failure (12). Several segments of the nephron are closely associated with sympathetic neuronal varicosities (4–7), and the highest number of neural fibers per tubule is found in the TAL (7). The TAL possesses both $\alpha_2$ (33)- and $\beta$-adrenergic receptors (16), and the selective $\beta$-adrenergic receptor agonist isoproterenol increases sodium reabsorption in the TAL (3). Thus renal nerve activity seems to be involved in the regulation of sodium reabsorption in TAL. In the present study, bilateral DNX prevented the excess sodium retention in CBL rats. This normalization of the sodium balance was associated with a significantly reduced expression of NKCC2 in the outer medulla in CBL rats and with normalization of the natriuretic response to furosemide. Together, these findings indicate that increased RSN activity known to be present in CBL rats plays a significant role in the formation of sodium retention by stimulating sodium reabsorption in the TAL.

We also examined the expression of NHE3 in the outer medulla. NHE3, which plays a major role in the regulation of urine acidification and might work as an alternative route for TAL sodium reabsorption, was significantly decreased in CBL rats. The mechanism behind this finding is unknown. However, DNX had no effect on the expression of NHE3 in both sham and CBL rats, indicating that changes in the abundance of this transporter are not affected by RSN.

CBL rats had, as was previously shown (20, 22), marked hypertrophy of the ISOM. Similar morphological changes are found in rats chronically treated with vasopressin (8, 10), and we have shown that this hypertrophy is absent in vasopressin-deficient Brattleboro rats with CBL-induced liver cirrhosis (22). Moreover, we have shown that chronic treatment with the somatostatin analog octreotide prevents the development of ISOM hypertrophy in CBL rats by an unknown mechanism (22). Despite the marked effect of DNX on TAL function in CBL rats, DNX did not prevent the formation of ISOM hypertrophy, which strongly indicates that RSN stimulation has functional but not hypertrophic action in the TAL.

Renal denervation increased the formation of solute-free urine in CBL rats. The final regulation of urine production is regulated by vasopressin and depends on 1) expression and membrane targeting of AQP2 in the collecting ducts and 2) the magnitude of the corticomedullary osmotic gradient generated by sodium reabsorption in the TAL. As was previously shown (23, 25), CBL rats had significantly decreased expression of AQP2. DNX had no effect on the abundance of AQP2 in either normal or CBL rats. However, because DNX prevented the formation of increased TAL sodium reabsorption, the formation of an increased corticomedullary osmotic gradient (21) most probably was prevented as well, resulting in the production of an increased amount of solute-free urine.

In summary, the present data indicate that RSN activity plays a significant role in the formation of sodium retention in CBL rats by stimulating sodium reabsorption in the TAL. An increasing number of reports support the hypothesis that regulation of TAL sodium reabsorption, including NKCC2 abundance, plays a significant role in different pathophysiological conditions with impaired renal sodium handling (1, 21, 31, 32, 35, 41). Detailed studies of the role of RSNs for the regulation of TAL function in these pathophysiological conditions, which include congestive heart failure, hypertension, and sepsis, are warranted.

The technical assistance of Iben Nielsen, Barbara Seider, and Haya Holmegaard is acknowledged.

This work received financial support from The Danish Medical Research Council, the Danish Heart Foundation, The Novo Nordic Foundation, The Eva and Robert Voss Hansen Foundation, The Ruth Køng-Petersen Foundation, and The Helen and Ejnar Bjørnov Foundation.

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