Portal hypertension induces sodium channel expression in colonocytes from the distal colon of the rat

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Fraser, Gerald M., Laurence M. Blendis, Patricia Smirnoff, Emanuel Sikular, Yaron Niv, and Betty Schwartz. Portal hypertension induces sodium channel expression in colonocytes from the distal colon of the rat. Am J Physiol Gastrointest Liver Physiol 279: G886–G892, 2000.—Cellular mechanisms for Na+ retention in portal hypertension are undefined, but epithelial Na+ channels (ENaC) may be involved. Under high-salt diet, ENaC are absent from distal colon of rat but can be induced by mineralocorticoids such as aldosterone. Presence of rat ENaC was determined by amiloride inhibition of 22Na uptake in colonocytes from the distal colon of the rat. This confirms that there was induction of rat ENaC and indicates that β-subunit mRNA was increased in PVL rats. Presence of mRNA transcripts, determined by RT-PCR, demonstrated that channel α- and γ-subunits were similarly expressed in both groups but that β-subunit mRNA was increased in PVL rats. Urinary Na+ was decreased for 3 days after PVL but was not different at other times, and serum aldosterone levels were elevated at 7 days, at a time when urinary Na+ output was similar to that of sham-operated rats. We conclude that PVL leads to induction of ENaC in rat distal colon. An increase in aldosterone levels may prevent natiuresis and is probably one of several control mechanisms involved in Na+ retention in portal hypertension.

Further vasodilatation causes additional increases in RAAS and sympathetic nervous system activity, which lead to the formation of ascites requiring diuretic therapy (33), diuretic-resistant ascites, and finally the hepatorenal syndrome. However, normal or suppressed plasma renin activity and serum aldosterone levels have been reported in cirrhotic patients with preascites or early ascites (4, 37, 38), and in as many as one-third of patients with well-established ascites, plasma renin activity and aldosterone levels are normal (2). This indicates that mechanisms other than the RAAS may be involved in Na+ retention.

The initial step in Na+ transport by salt-absorbing epithelia is entry into the cell across the apical membrane. The Na+ channel is one of the important membrane transport proteins responsible for this process and is found in many tissues, including colon, kidney, lung, and sweat glands (19). Important functional characteristics of this channel include inhibition by substituted pyrazine derivatives such as amiloride and upregulation by hormones such as aldosterone and antidiuretic hormone (19).

The cellular mechanisms for absorption of Na+ in the colon vary according to species and segment (32). Under normal conditions, the dominant mechanism for Na+ absorption in the distal colon of the rat is Na+/H+ exchange (5, 31). Following treatment of rats with mineralocorticoids, Na+ absorption becomes electrogenic (6, 17) because of activation and induction of Na+ channels (3). In previous studies, we used ion flux (18) and patch clamp (15) techniques to determine the distribution of Na+ conductances in crypts from corticosteroid-treated rat distal colon. We found that Na+ conductance induced by corticosteroids decreased gradually from the luminal surface to the base of the crypt. Full activity of the Na+ channel requires the presence of three subunits (α, β, and γ) (8). Expression of the

DURING THE PROGRESSION OF cirrhosis, there is an increase in vascular capacity due to peripheral arterial vasodilatation starting in the splanchnic vascular bed. The physiological response to the imbalance between vascular capacity and intravascular volume is stimulation of Na+-retaining neurohumoral systems, especially the renin-angiotensin-aldosterone system (RAAS) and the sympathetic nervous system activity. This results in Na+ retention until a new steady state is reached (40).
α-subunit alone in *Xenopus* oocytes demonstrated a conductance sensitive to amiloride and highly selective for Na⁺, as expected for a Na⁺ channel, although the current was low (7). Two additional subunits (β and γ) were subsequently cloned, and they increased current by 100-fold when functionally expressed in oocytes together with the α-subunit (8). In untreated animals, we were unable to detect rat epithelial Na⁺ channel (rENaC) subunit mRNA by Northern blot, but following treatment with dexamethasone for 3 days, mRNA was induced for all three subunits (18).

Prehepatic portal hypertension following partial ligation of the portal vein in the rat leads to a series of hemodynamic events (1, 11, 25, 34). These rats develop increased portal venous inflow and a hyperdynamic systemic circulation. Although the hyperdynamic circulatory pattern persists for at least 8 days (35), no significant difference in intestinal hemodynamics was found at 10 days (22). Furthermore, despite persistence of portal hypertension, the systemic hyperdynamic circulation disappears by 6 mo (35). It was subsequently shown that immediately after induction of portal hypertension, rats develop a hyperdynamic circulation, a fall in systemic vascular resistance, and a transient fall in central blood volume within 24 h (1, 11). Then, within the next 24 h, the total body exchangeable Na⁺ increases and remains increased for at least 8 days (11). The role of nitric oxide in the pathogenesis of vasodilation and Na⁺ retention is unclear (25, 27), although Na⁺ retention has been shown to vary with liver dysfunction (28). Despite Na⁺ retention and an expanded Na⁺ space, these animals never develop ascites.

In the present study, we investigated the effect of portal hypertension on Na⁺ transport in the rat distal colon. In particular, the physiological activity of the rENaC and presence of mRNA for the α-, β-, and γ-subunits are examined.

**METHODS**

**Experimental animals.** Male Charles River-derived (inbred) rats were purchased from the Levenstein Animal House (Yokneam, Israel) and divided into different experimental groups. The study was approved by the Animal Welfare Committee of the Faculty of Agriculture of the Hebrew University of Jerusalem, Israel.

**Portal vein ligation and sham-operated rats.** Rats weighing 240–260 g were subjected to portal vein ligation (PVL) or a sham operation. The animals were anesthetized under slight ether-ethanol (2%) solution (J. T. Baker, Phillipsburg, NJ). The portal vein was isolated at laparotomy, and a single 3-0 silk ligature was tied around both the portal vein and a 20-gauge blunt-tipped needle. The needle was then removed, and the portal vein was allowed to reexpand distal to the stenosis. In the sham animals, the portal vein was dissected free but no ligature was placed. The rats were allowed to recover in their cages and were fed with standard rat chow (Kofolk, Petach-Tikva, Israel) containing a 0.12 mmol Na⁺/g feed. Designated groups of rats were kept in metabolic cages for Na⁺ balance studies. A separate group of rats fed standard chow was used to establish the normal range of plasma levels of aldosterone (controls).

**Na⁺ output in urine and feces.** We performed Na⁺ balance studies to determine the timing of changes in Na⁺ excretion in the kidney and gastrointestinal tracts following PVL. The animals were individually housed in metabolic cages at 25 ± 4°C with a 12:12-h light/dark cycle. The animals had free access to water and to rat chow containing 0.12 mmol Na⁺/g feed. Balance studies were performed on four sham and four PVL rats for 3 days before surgery and 13 days after surgery. The day of surgery was day 0. Daily urine and stool samples were small, and collections were obtained in blocks for the 3 presurgical days and days 1–3, 4–6, 7–10, and 11–13 after surgery. The combined specimens in each block were analyzed for their Na⁺ content.

Na⁺ concentrations in urine and feces were determined by inductively coupled plasma atomic emission spectrometry. Samples were prepared for analysis by microwave-assisted digestion by using an MLS 1200 mega microwave digestion unit (Milestone Sorisole). Five milliliters of each urine sample or 250 mg of dry stool were transferred into 50-ml polypropylene disposable test tubes, and 5 ml of concentrated nitric acid were added. Each tube was fitted with a lightly tightened screw cap. The tubes, in a plastic stand, were transferred to the temperature-controlled microwave oven, and the samples were subjected to two digestion cycles of 20 min each, at 450 W of microwave power and 95°C. The vessels were allowed to cool for 10 min between cycles, and at the conclusion of the digestion program they were brought to room temperature and were uncapped. The volume was made up to 25 ml with deionized water.

Analyses were conducted on portions of these solutions, versus multi-element standards, prepared using the same matrix. An ICP Spectroflame (Spectro, Kleve, Germany), equipped with a standard cross-flow nebulizer, power level of 1.2 kW, coolant flow of 15 l/min, auxiliary flow of 0.5 l/min, and nebulizer flow of 0.5 l/min, was used.

**Measurement of portal pressure.** Portal venous pressure was determined before and after partial PVL by measurement of intrapressure pressure (n = 9). Animals were anesthetized as previously described, and a No. 22 disposable needle was introduced into the splenic pulp at laparotomy. The needle was connected to a three-way tap to enable perfusion of the system through one arm and to a transducer set at the level of the right atrium through the other arm. The needle was flushed with saline to create a small saline droplet around the end of the needle within the splenic pulp. The pressure was then recorded continuously on a Gilson polygraph (Middleton, WI). Three separate pressure recordings were taken, and the mean for each animal was calculated.

**Preparation of colonic cell populations and measurement of amiloride-sensitive ²²Na⁺ uptake.** Colonocytes were isolated by a Ca²⁺ chelation method, and amiloride-sensitive ²²Na⁺ uptake was determined as previously described (18). The rats were killed by cervical dislocation, and the distal colon was rapidly isolated, removed, and cleansed. The colon was everted, and one end was ligated and filled with isolation medium followed by ligation of the other end to form a closed loop. The distended loops, with the luminal surface exposed, were immersed and shaken in the dissociation buffer containing MEM medium supplemented with FCS (Beit Haemek Biological Industries), dithiothreitol, and Ca²⁺ chelators (EDTA) at the appropriate concentration ranges. The medium was replaced at selected time intervals with fresh dissociation buffer. Five cell populations were harvested stepwise from the surface to base of the crypts and designated C1 to C5.
The combined luminal cell population (C1 + C2) was washed in HEPES buffer (in mM: 127 NaCl, 5.9 KCl, 2.1 KH₂PO₄, 2.5 CaCl₂, 1.2 MgCl₂, 11.5 glucose, 0.3 l-glutamine, and 25 HEPES, pH 7.4) with 1 mM dithiothreitol and resuspended in 1 ml HEPES buffer with 1% BSA. After centrifugation at 800 g, the pellet was resuspended in 1 ml HEPES-BSA buffer and divided into three equal portions. Buffer containing 0.3 nCi ²²Na⁺ was added in the presence or absence of 10 μmol amiloride. The cells were incubated for 10 min at 37°C while being agitated. At the end of the reaction, 1.5 ml of iso-osmolar N-methyl-D-glucamine (NMDG) buffer (in mM: 140 NMDG, 3 KCl, 1 CaCl₂, 1 MgCl₂, 10 glucose, and 10 HEPES) was added, the reaction mixture was centrifuged at 800 g for 5 min, and the supernatant was discarded. The cell pellet was resuspended in 400 μl cold NMDG buffer and loaded over 200 μl of phthalic acid dinonyl ester oil. Following centrifugation at 1,100 g, the aqueous and oily supernatants were removed, and the cell pellet was dissolved in 500 μl of methylbenzethonium (hydroxy hyamine 10-X). The intracellular ²²Na⁺ activity was counted by scintillation spectrometry. Results are expressed as nanomoles per milligram protein for the 10-min incubation period.

**Protein determination.** Protein concentration in the different suspensions is determined by a Pierce kit (23) employing a microbicinchonic acid-based protein assay and BSA as the protein standard.

**Serum aldosterone.** Serum aldosterone concentrations were measured by solid-phase radioimmunoassay (Diagnostic Products, Los Angeles, CA) in blood samples obtained from controls and from sham and PVL rats at 7 and 14 days.

**RNA isolation.** Total RNA was extracted from colon mucosal scrapings (luminal colonocytes) according to a protocol for single-step RNA isolation based on acid guanidinium-thiacyanate-phenol-chloroform extraction, using TriReagent (Molecular Research Center, Cincinnati, OH) solution (9). Total RNA was aliquoted and quantified in sterile tubes.

**RT-PCR reaction and quantitative evaluation for rENaC α-, β-, and γ-subunits.** The whole isolated RNA was reverse transcribed and amplified with the Access RT-PCR system (Promega). The RT-PCR of the isolated RNA was performed using appropriate primers, reverse transcriptase, and DNA polymerase enzymes. The concentrations of RNA from different origins were adjusted to the same level using β-actin as a standard. The cDNA specific for the α-, β-, and γ-subunits of the ENaC channel subunit genes was amplified using sequence-specific oligonucleotide primers giving rise to products of 369, 648, and 130 bp for α-, β-, and γ-subunits, respectively and a product of 369 bp for β-actin. The α-, β-, and γ-subunit PCR primers consisted of bases 96–116, 69–89, and 361–381 in the sense direction, respectively (5'-CTAGACCTTTCAGCCTCCCAAC-3', 5'-CACCATGGCGATGGAGAGA-3', and 5'-ACTCCAGAACTGATTTC-3'), and bases 460–440, 2041–2021, and 491–510 in the antisense direction, respectively (5'-AGTCTCAGGGCAGAAGAGAC-3', 5'-CACATCCTGAAGCTCAAG-3', and 5'-AGTCTCAGGTTT-3'), and bases 359–341 (5'-AACCGCAGAGAATGACCCAGATG-3') and in the antisense direction consisted of bases 705–734 (5'-AGACAGGCTGCGCATCTTGTGCGGATGTC-3'). The RT-PCR reaction mixture contained 1 μl of H₂O-diluted RNA (1 μg/μl), 10 μl AMV/Tfi 5× Reaction Buffer, 1 μl dNTP (10 mM each dNTP), 1 μl downstream primer (50 μM), 1 μl upstream primer (50 μM), 2 μl 25 mM MgSO₄, 1 μl Tfi DNA polymerase (50 U/μl), and nuclelease-free water to a final volume of 40 μl. The RT-PCR program consisted of one cycle at 48°C for 1 h followed by one cycle at 94°C for 2 min for reverse transcription. The PCR amplification was performed by 35 cycles for α-, β-, and γ-subunits or 30 cycles for β-actin of 45 s at 94°C, 1 min at 56°C, and 1 min at 72°C and a final extension at 72°C for 7 min.

The resulting RT-PCR products were submitted to electrophoresis in 2% agarose gel for α- and γ-subunits and β-actin products and 1% agarose for β-subunit products. The cDNA products were detected with ethidium bromide by ultraviolet illumination. The signals of the electrophoretic assay were quantified using a densitometer scanner with the Fujix PhosphorImager apparatus and the BAS-1000 Bio-Imaging Analyzer (Fuji Photo Film). Results of the densitometric determinations for the α-, β-, and γ-subunits of the ENaC were corrected according to the corresponding β-actin gene transcription. Six independent determinations were performed.

**Data and statistics.** Data are means ± SD unless stated otherwise, and Student’s t-test was used to determine the significance of differences between experimental groups.

**RESULTS**

**Portal venous pressure.** To confirm that in our hands PVL significantly increased portal venous pressure, the intrapleural pulse pressure (portal venous pressure) was measured in 10 rats before and after partial PVL. Pressure increased by 5.0 ± 0.5 mmHg.

**Na⁺ balance and serum aldosterone.** There was no significant difference between sham and PVL rats in body weight, stool weight, or urinary volume at any of the time intervals. The daily intake of Na⁺ was 1.7 mmol and was calculated from the average daily intake of chow and the known Na⁺ content. The mean daily urinary output of Na⁺ was 0.35 ± 0.11 mmol/day in PVL rats compared with 0.89 ± 0.27 mmol/day in sham rats during the first 3 days after surgery (P = 0.01) (Fig. 1A). No significant differences were noted for any of the other periods. Total mean urinary Na⁺ excretion for the 13-day follow-up period was 20.21 mmol in sham rats compared with 18.66 mmol in PVL rats, and this difference was entirely accounted for by the 1.62 mmol difference between the two groups during days 1–3. Small, nonsignificant differences were noted in the remaining periods. Total mean fecal Na⁺ output in sham and PVL rats was 2.62 mmol and 2.07 mmol, respectively. Although no significant differences were noted during any of the four follow-up periods, there was a trend toward lower fecal Na⁺ excretion in PVL rats on days 7–10 (P = 0.09) (Fig. 1B). The PVL rats remained in positive Na⁺ balance [diary intake – (urinary Na⁺ + fecal Na⁺)] throughout the postsurgical period, whereas in the sham rats the Na⁺ retained during days 1–3 was subsequently excreted (Fig. 1C).

**Serum aldosterone concentrations were determined in blood samples obtained from control (n = 7), sham (n = 11), and PVL (n = 12) rats at 7 days and from sham and PVL rats at 14 days. The mean level in the controls was 654.8 ± 72.4 nmol/ml. Aldosterone levels were significantly increased in PVL rats compared with sham rats at 7 days (873.8 ± 141.8 nmol/ml and 1,078.8 ± 108.4 nmol/ml, respectively; P < 0.0001) but not at 14 days (890.1 ± 59.7 and 859.0 ± 90.5 nmol/ml, respectively; P = 0.4).
Amiloride-sensitive Na\(^+\) uptake. Amiloride-sensitive Na\(^+\) uptake was determined as the difference in \(^{22}\)Na\(^+\) uptake by isolated luminal colonocytes in the presence and absence of 10 \(\mu M\) amiloride (Fig. 2). Inhibition of Na\(^+\) uptake was increased in colonocytes from PVL compared with sham rats at both 7 and 14 days. Uptake in sham and PVL rats was 0.77 ± 0.83 (\(n = 11\)) and 3.36 ± 2.01 (\(n = 11\)) nmol/mg protein at 7 days (\(P < 0.0001\)) and 0.06 ± 0.02 (\(n = 3\)) and 0.92 ± 0.57 (\(n = 6\)) nmol/mg protein after 14 days (\(P = 0.04\)).

RT-PCR for rENaC α-, β-, and γ-subunit mRNA. The electrophoretic pattern of α-, β-, and γ-subunit cDNA, obtained after reverse transcription of RNA extracted from sham and PVL rat colonocytes at 7 days, is shown in Fig. 3. The housekeeping gene β-actin was used to ensure RNA quality and equal loading. The densitometry analysis for six gels is summarized in Fig. 4. The values are expressed in arbitrary units normalized with respect to β-actin. Expression of α- and γ-subunit mRNA was similar in the two groups, whereas β-subunit mRNA was low in sham animals but markedly increased in PVL rats.

DISCUSSION

In this study, we examined the ENaC as a possible mechanism for the Na\(^+\) retention in portal hypertension. Na\(^+\) retention is an important consequence of portal hypertension, but the cellular mechanisms responsible for this process are not known. We used the PVL rat, which is a model in which no ascites develop. Since patients with preascitic portal hypertension may have elevated, normal, or depressed blood levels of aldosterone (4), it was of interest to determine whether rENaC would be induced in the setting of this rat model. Amiloride-sensitive Na\(^+\) uptake was measured in surface epithelial cells from the distal colon with the use of techniques that we have previously described (20). The rationale for using colonocytes was based on the observation that the rat distal colon does not normally express rENaC, but following exposure of the colon to mineralocorticoids such as aldosterone, functioning rENaC are detected (19).

Na\(^+\) channels in surface colonocytes were detected by inhibition of \(^{22}\)Na\(^+\) uptake by 10 \(\mu M\) amiloride in PVL in surface colonocytes at 7 and 14 days. In sham rats, uptake inhibition was low at 7 days and absent at
14 days. In PVL rat channels, inhibition of uptake was significantly greater at 7 than at 14 days and significantly greater than in sham rats at both intervals. We believe that the presence of rENaC at 7 days in sham rats may have been induced by the stress of surgery.

\[ ^{22}\text{Na}^{+}\text{ uptake inhibition in PVL rats at 7 days was 19% of that induced by treatment with dexamethasone (18)} \]

Thus partial PVL leads to submaximal induction of rENaC.

The decrease in amiloride-sensitive \( ^{22}\text{Na}^{+} \) uptake in sham and PVL rats at 14 days compared with 7 days suggests that the factors responsible for induction of Na\(^{+}\) channels decrease after surgery. However, there are differences between the two groups. Sham rats returned to Na\(^{+}\) balance shortly after surgery, but PVL rats remained in positive Na\(^{+}\) balance throughout the study period. In addition, PVL rats demonstrated measurable amiloride-sensitive \( ^{22}\text{Na}^{+} \) uptake at 14 days compared with negligible activity in the sham group. Thus the factors responsible for induction of channel activity probably remain elevated in PVL rats at 14 days, and rENaC contributes to the expanded Na\(^{+}\) space previously described in this model (12).

To further investigate the effect of PVL on the expression of rENaC, we determined the levels of mRNA for the \( \alpha\)-, \( \beta\)-, and \( \gamma\)-subunits by PCR. We were able to show that ENaC \( \alpha\)- and \( \gamma\)-subunit mRNA was present in equal amounts in PVL and sham-operated rats. \( \beta\)-Subunit mRNA was markedly increased in PVL rats, which is consistent with the concept that the \( \beta\)-subunit is involved in the regulation of ENaC expression in the rat distal colon. Full activity of the Na\(^{+}\) channel requires the presence of three subunits (\( \alpha\), \( \beta\), and \( \gamma\)) (8).

Several techniques have been used to map the presence of rENaC subunit mRNA and protein in rat distal colon after treatment with corticosteroids, including in situ hybridization (14), Northern blot, and immunohistochemistry (14, 30). After corticosteroid challenge, the \( \alpha\)-subunit was found to be constitutively expressed, whereas the \( \beta\) and \( \gamma\)-subunits were induced.

We also investigated the relationship of serum aldosterone levels to the induction of rENaC and Na\(^{+}\) excretion. Serum aldosterone levels were significantly elevated in PVL rats compared with sham rats at 7 days, which coincided with maximal amiloride-sensitive Na\(^{+}\) uptake in the colonocytes. However, at 14 days there was no difference in aldosterone levels,
although uptake was still significantly increased in PVL compared with sham rats. This provides circumstantial evidence that aldosterone was involved in the induction of the channels but does not exclude additional control mechanisms.

Partial ligation of the portal vein in the rat leads to a series of hemodynamic events in the splanchnic and system circulations (1, 11, 12). These rats develop a hyperdynamic systemic circulation, as described in patients, with a fall in systemic vascular resistance and a transient fall in central blood volume within 24 h. During the next 24 h, the total body exchangeable Na⁺ increases (Na⁺ space) and reaches a maximum at 4 days, which is associated, interestingly, with increased renal blood flow. Finally, there is an increase in mesenteric blood flow. These hemodynamic changes persisted for up to 10 days but completely disappeared by 6 mo despite persistent portal hypertension (11). Our Na⁺ balance studies are consistent with this sequence of changes. We found that urinary Na⁺ output was significantly lower in PVL compared with sham rats in the 3 days immediately after surgery, which is consistent with changes in total body Na⁺ previously described (1, 11). However, at 7 days, when serum aldosterone levels were elevated in PVL rats, no difference in urinary Na⁺ excretion was noted. It is possible that aldosterone was important in maintaining positive Na⁺ balance and prevented compensatory diuresis.

Changes in Na⁺ transport in the colon following PVL may provide clues to the mechanism for Na⁺ retention by the kidney in portal hypertension. Early studies showed that the cortical collecting ducts possess an electroneutral Na⁺/K⁺-dependent transport mechanism for Na⁺ (36). More recent patch clamp studies have confirmed this finding. A low-conductance channel (3–5 pS) with prolonged open and closed states and high selectivity for Na⁺ over K⁺ was described (26, 29), and this channel is identical to the rENaC found in rat distal colon. rENaC has been found in additional areas of the distal nephron, including the outer medullary collecting duct, the distal convoluted tubule, connecting tubule, and proximal tubule (10, 20, 39).

The presence of rENaC α-, β-, and γ-subunit mRNA has been confirmed in the kidney by Northern blot and immunohistochemical methods (30). Northern blot analysis for subunit mRNA in kidney cortex showed slight induction of α-rENaC following administration of either dexamethasone or aldosterone and no change in β- and γ-rENaC (3). Following adrenalectomy, there was a decrease in α-subunit mRNA as determined by RNase protection assay but no decrease in β- and γ-subunits. This deficit was not corrected after 2 days but was corrected after 7 days of infusion of aldosterone (16). However, in cultured cortical collecting duct cells exposed to aldosterone, there was an increase in γ-subunit-specific mRNA as determined by quantitative PCR (13). Thus the kidney appears to differ from colon in regulation of channel expression, and further studies will be needed to investigate the effect of portal hypertension on ENaC in this organ.

In conclusion, we have shown that partial PVL induces rENaC in rat distal colon, and this is associated with an increase in serum aldosterone levels. Urinary output of Na⁺ is reduced in the 3 days after ligation but at 7 days is similar to sham rats despite elevated serum aldosterone. Regulation of rENaC is controlled, at least in part, by changes in levels of β-subunit mRNA. The relevance of these changes to Na⁺ retention by the kidney in portal hypertension and the putative role of nitric oxide in this regard require further study.

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