Altered expression of renal apical plasma membrane Na\(^+\) transporters in the early phase of genetic hypertension

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Capasso, Giovambattista, Maria Rizzo, Ciriana Evangelista, Patrizia Ferrari, Ghislaine Geelen, Florian Lang, and Giuseppe Bianchi. Altered expression of renal apical plasma membrane Na\(^+\) transporters in the early phase of genetic hypertension. Am J Physiol Renal Physiol 288: F1173–F1182, 2005.—The present study explores whether the development of hypertension in the Milan strain of rats (MHS) rats is preceded or paralleled by alterations of mRNA and/or protein levels of the major luminal Na\(^+\) transporters. MHS rats were studied at 23–25 days after birth; age-matched Milan normotensive (MNS) rats were used as controls. The glomerular filtration rate (GFR), measured by inulin clearance, was higher in MHS than in MNS rats, while the mean blood pressure was not different in the two strains of animals indicating that the MHS rats were still in the prehypertensive state. Type 3 sodium/hydrogen exchanger (NHE3), bumetanide-sensitive sodium-potassium-2 chloride cotransporter (NKCC2), sodium-chloride cotransporter (NCC) and \(\alpha\)-ENaC mRNA abundances were quantified by competitive PCR. In MHS compared with MNS, mRNA abundance was unchanged for NHE3 in proximal tubules, higher for NKCC2 in medullary thick ascending limbs of Henle’s loops (TAL) and lower for NCC in distal convoluted tubules (DCT) and for \(\alpha\)-ENaC along collecting ducts (CD). Western blot experiments revealed 1) unchanged NHE3; 2) a significant increase in NKCC2 in the outer medulla; 3) a significant decrease in NCC in the renal cortex and of \(\alpha\)-ENaC in both the renal cortex and outer medulla, whereas \(\beta\)- and \(\gamma\)-ENaC remained unchanged. These data indicate that, in MHS rats, there is a strong upregulation of NKCC2 along the TAL associated with increased GFR, robust inhibition of NCC cotransporter along the DCT and modest downregulation of \(\alpha\)-ENaC along the CD. The interplay of the various Na\(^+\) transporters may well explain why, at this age, the rats are still in the prehypertensive state.

Hypertension is a major risk factor for heart disease, stroke, and renal failure. Although its pathogenesis is unknown in the majority of patients, it is becoming evident that the kidney may play an important role in both the induction and maintenance of many types of systemic hypertension. Accordingly, hypertension accompanies the kidney when this organ is transplanted from a genetically hypertensive into a normal rat (5). In addition, recent human genetic studies demonstrate that mutations of genes, encoding for proteins expressed in the kidney and involved in tubular ion transport, are associated with modifications of systemic blood pressure. For instance, loss-of-function mutations of transport molecules in the thick ascending limb of Henle’s loop (TAL) lead to Bartter’s syndrome, and a defective thiazide-sensitive sodium-chloride cotransporter (NCC), present in the distal tubule, is the cause of Gitelman’s syndrome (40). The modifications of these ion-transporting systems are characterized by urinary sodium loss, resulting in orthostatic hypotension. In contrast, gain-of-function mutations of amiloride-sensitive sodium channels in the collecting ducts generate Liddle’s syndrome, which is phenotypically characterized by systemic hypertension (41). Studies on animal models for genetic hypertension have also highlighted the importance of the kidney in the development of the hypertension. In particular, in the Milan hypertensive strain of rats (MHS), hypertension develops because of a primary alteration in renal tubular sodium reabsorption (5). It has been shown that induction of hypertension is preceded by a phase of salt retention due to increased tubular sodium reabsorption. Subsequent experiments have also clarified that the primary event explaining the enhanced tubular sodium reabsorption is the increased activity and expression of Na\(^+\)-K\(^+\)-ATPase (33).

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ference in NHE3 expression and protein abundance between the two strains but that MHS animals show significantly increased expression, along the medullary TAL, of the NKCC2 gene at both the mRNA and protein levels. Interestingly, NCC gene transcript abundance, measured in distal convoluted tubules, and related protein abundance were significantly reduced in MHS. Finally, the abundances of mRNA and protein of α-ENaC were also decreased in both the cortical and outer medullary collecting ducts in MHS, whereas β- and γ-ENaC protein abundances were unaffected.

**METHODS**

The experiments were performed using MHS and MNS strains of rats, aged 23–25 days. All the investigations involving animals were conducted in conformity with the Guiding Principles in the Care and Use of Laboratory Animals of the American Physiological Society and were approved by the Ethical Committee of the Second University of Naples.

**Measurement of GFR.** The animals were anesthetized [ketamine (60 mg/kg body wt) plus xylazine (10 mg/kg body wt), and the right femoral artery was catheterized (PE-10 tubing) to record blood pressure and to obtain blood for measurements of inulin. The left femoral vein was cannulated and used for intravenous (iv) infusion via a syringe pump (Braun Apparatus) of a modified Ringer-saline solution containing inulin (0.1 mg/ml; Inutest polyfructosan; Boehringer-Mannheim, Mannheim, Germany). The two catheters were externalized through a small incision made in the skin at the back of the head. They were kept filled by flushing them with heparinized solution to prevent clotting. Four hours after the surgery, when the rats were completely recovered from anesthesia, a priming dose of inulin (0.4 ml/100 g body wt) was infused followed by a maintaining dose at 0.6 ml·h⁻¹·100 g body wt⁻¹. After an equilibration period of 60 min, three arterial blood samples (20 μl) were taken every 20 min. According to Earle and Berliner (13), under these conditions the infusion rate of inulin will equal the excretion rate and therefore the GFR may be calculated as:

\[
\text{perfusion rate} \times \frac{\text{plasma inulin}}{\text{perfusion inulin}}
\]

where perfusateinulin and plasmainulin are the inulin concentrations in perfusate and plasma, respectively.

Mean arterial blood pressure was measured in conscious rats through the femoral artery catheter connected to a pressure transducer (XCDR-P33 XL, Gould, Cernusco-Milan, Italy) attached to a multichannel recorder (Gould Instrument System, RS 3800).

**Plasma osmolality, AVP, and aldosterone measurements.** Trunk blood was carefully collected after decapitation of the animals. Plasma osmolality was measured on 20-μl samples using a Fiske One-Ten osmometer. For plasma AVP measurement, the extraction procedure was miniaturized from that used by Skowesky et al. (42), as previously described (19), using 700-μl plasma samples, with adsorption of the hormone onto bentonite followed by an elution using acidified acetone (80% acetone-20% IN HCl). Intra- and interassay coefficients of variation were 7 and 13.5%, respectively. AVP was then measured by radioimmunoassay using antisera K9-IV (gift of Dr. L. C. Keil, NASA Ames Research Center, Moffett Field, CA) (25), as previously described (19), synthetic (375 IU/mg, gift of Ferring, Malmö, Sweden), and 125I-labeled iodo-AVP prepared in our laboratory. The assay has a sensitivity of 0.25 pg/assay tube, an ED50 of 1.7 pg AVP, and a useful range up to 50 pg/assay tube.

For the aldosterone measurements, the rats were anesthetized with ether, the abdomen was opened, and the blood was drawn from the abdominal aorta. Blood collection was interrupted as soon as dyspnea appeared. Plasma aldosterone concentrations were determined using a commercially available radioimmunoassay kit (ALDOCTK-2, Sorin P2714, Sorin Medica, Saluggia, Italy).

**Tabule microdissection.** After anesthesia [ketamine (60 mg/kg body wt) plus xylazine (10 mg/kg body wt)], the left kidney was removed and washed in ice-cold dissection solution containing (in mM) 137 NaCl, 5.4 KCl, 25 NaHCO₃, 0.3 Na₂HPO₄, 0.4 KH₂PO₄, 0.5 MgCl₂, 10 Hepes, 5 glucose, and 1 glycine. Proximal and distal convoluted tubules, medullary TAL, and cortical and outer medullary collecting ducts were isolated according to the method described earlier by Schafer et al. (39). Small pieces of renal cortex (for proximal tubules, distal tubules, and cortical collecting ducts) and inner stripe of outer medulla (for TAL and outer medullary collecting ducts) were cut under the stereomicroscope and incubated for 30 min at 37°C in the microdissection solution containing 0.5 mg/ml collagenase, continuously bubbled with 95% O₂-5% CO₂. After digestion, the tissues were washed with ice-cold collagenase-free dissection solution containing 1 g/l albumin. Proximal tubules, distal tubules, and cortical collecting ducts were isolated free hand from the renal cortex under the stereomicroscope; distal tubules were identified by their appearance among the other superficial tubules of the renal cortex. TAL were separated from other cells and fragments by filtering the supernatant through a 80-μm-opening nylon mesh (Milipore). The use of collagenase eased the tubular dissection, thus allowing harvesting of enough material for the molecular biological experiments.

**RNA purification.** Total RNA (0.5–2 μg) was purified from proximal tubules, TAL, distal tubules, and collecting ducts. After isolation, the tubules were transferred into 350 μl of lysis buffer containing guanidinium isothiocyanate and β-mercaptoethanol. The lysate material was loaded on a silica gel membrane (Qiagen) that specifically binds total RNA. Possible contamination from genomic DNA was prevented by incubation of the membrane with 30 U of RNase-free DNase I (Qiagen) for 15 min at 25°C. The membrane was washed three times, and total RNA was eluted in 50 μl of 1 g/l diethylpyrocarbonate (DEPC-water). The concentration and purity of RNA were determined by measuring its absorbance at 260 and 280 nm using a GeneQuant RNA/DNA calculator (Pharmacia Biotech).

**Reverse transcription.** cDNA was synthesized from equal amounts of total RNA (100–200 ng) using 200 U murine leukemia virus reverse transcriptase (GIBCO BRL), 0.5 μg of oligo(dT)₁₂-₁₈ (GIBCO BRL), 10 mM dithiothreitol, and 2.5 mM dNTP (Pharmacia) in a total volume of 20 μl. Before the addition of reverse transcriptase, the reaction mixture was incubated at 70°C for 3 min to denature the RNA, then cDNA was synthesized at 37°C for 1 h. Controls, incubated as above, but without addition of reverse transcriptase, were included in each run.

**PCR.** PCR reactions were performed in a total volume of 50 μl in the presence of: 10 pmol of each oligonucleotide primer, 200 μM dNTP solution, 5 μl of 10× PCR buffer, 1.5 mM MgCl₂, and 1.25 U Taq polymerase. Primer sequences are listed in Table 1. The expected sizes of the PCR products were 622 bp for NHE3, 603 bp for NKCC2, 550 bp for NCC, and 678 bp for α-ENaC. Samples were denatured at 95°C for 3 min, followed by 33 cycles consisting of denaturing at 95°C (1 min), annealing at 65, 60, 58, and 60°C (30 s) for NHE-3, NKCC2, NCC, and α-ENaC, respectively, and extension at 72°C (1 min). After amplification, PCR products were subjected to size separation by agarose gel electrophoresis (18 g/l).

**Internal standard preparation.** cDNA, synthesized as described before, was utilized for internal standard synthesis. For the internal standard, the sequences of antisense primers are listed in Table 1. The expected sizes of internal standards were 546 bp for NHE3, 520 bp for NKCC2, 453 bp for NCC, and 605 bp for α-ENaC.

All the PCR reactions were performed as described before. The various products were analyzed by agarose gel electrophoresis (18 g/l), and they were recovered from the gel using an agarose gel DNA extraction kit (Clontech). The concentration and purity were determined by measuring the absorbance at 260 and 280 nm.
Table 1. Primers used in competitive PCR

<table>
<thead>
<tr>
<th>Sense Primer</th>
<th>Antisense Primer</th>
<th>Linker Antisense</th>
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<tbody>
<tr>
<td>NHE3</td>
<td>5'-ACGAAAGAACAGACTATGCC-3'</td>
<td>5'-GGGATCTAATCGCCTGACGTTTAA-3'</td>
</tr>
<tr>
<td>NKCC2</td>
<td>5'-GGCCTCTATAGGCGCTTTA-3'</td>
<td>5'-AGGTGGTGCCCTCATTTCTG-3'</td>
</tr>
<tr>
<td>α-ENaC</td>
<td>5'-ATGGACGCTGACGACAGGAA-3'</td>
<td>5'-GGGATCTAATCGCCTGACGTTTAA-3'</td>
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NHE3, isoform 3 of Na+/H+ exchanger; NKCC2, bumetanide-sensitive Na+/K+2Cl– cotransporter; NCC, thiazide-sensitive Na+/Cl– cotransporter; α-ENaC, epithelial sodium channel α-subunit.

Quantification of NHE3, NKCC2, NCC, and α-ENaC mRNA. For each mRNA quantification, competitive PCR reactions were performed using as templates the cDNA obtained from total RNA, extracted from the various tubular segments, and the corresponding internal standards. The NHE3-specific primers yielded products of 622 and 546 bp for wild-type and internal standard, respectively. Two PCR products of NKCC2-specific primers produced fragments of 603 and 520 bp for internal standards. The NHE3-specific primers yielded products of 550 (wild-type) and 453 bp (internal standard) were yielded by NCC-specific primers. For α-ENaC, the PCR products were of 678 and 605 bp. Five to eight competitive PCRs were performed by addition of decreasing amounts of the competitive template (internal standard) to replicate reactions containing identical amounts of cDNA. A progressive decrease in the competitive template PCR product corresponds to a progressive increase in the wild-type template PCR product. The PCR products were separated on 18-g/l agarose gels and stained with ethidium bromide. The gel was photographed, and fluorescence intensity of PCR products was quantified using NIH Image 1.60. The amounts of NHE3, NKCC2, NCC, and α-ENaC mRNA were calculated using a log-log scale plot of the ratio of PCR products vs. the known amount of internal standard used in the competitive PCR reactions. When the wild-type and competitive PCR products were equivalent [log (ratio) = 1], the amounts of wild-type NHE3, NKCC2, NCC, and α-ENaC mRNA present in the initial sample were equal to the known starting amount of the competitive template. Results are expressed per nanogram of total RNA.

Western blot analysis of NHE3, NKCC2, NCC, and β-ENaC subunit protein abundances. Thin pieces (100–200 mg) of renal cortex (for NHE3, NCC, and ENaC subunits) and of inner stripe of outer medulla (for NKCC2 and ENaC subunits) were frozen at –80°C and then disrupted with a Potter homogenizer at 4°C in membrane buffer (150 mM NaCl, 50 mM Tris-HCl, 5 mM EDTA) containing (in µg/ml) 50 4-(2-aminoethyl)-benzenesulfonyl fluoride, 2 leupeptin, and 2 aprotinin.

After the centrifugation at 10,000 g for 15 min at 4°C, the supernatant was determined by Bradford assay.

The same approach was used to quantify the transcripts of the NHE3, NKCC2, NCC, and ENaC subunit transport proteins.

RESULTS

In Table 2, the data on renal hemodynamics, mean arterial blood pressure, osmolality, AVP, and aldosterone plasma levels are reported. As already shown in previous papers (17, 37), at this age the GFR, relative to kidney weight, was substantially increased in MHS compared with MNS animals; moreover, no significant difference in systemic blood pressure was found between the two strains of rats; i.e., the MHS rats were still in the prehypertensive stage. With respect to plasma osmolality and AVP concentration levels, the results did not show any significant difference between MNS and MHS rats. The same was found for aldosterone, thus confirming previous results (31).

NHE3 mRNA and protein abundance along the proximal tubule and TAL. Figure 1A shows the typical gels of competitive PCR for NHE3 (representative of 7–8 experiments): the addition of decreasing amounts of internal standards resulted in a corresponding increase in wild-type template products. Figure 1B shows the corresponding log-log plots. The amounts of wild-type mRNA are calculated when the intensity of internal standard and wild-type PCR products are equated (ratio = 1). The same approach was used to quantify the transcripts of the other Na+ transport proteins.

Table 2. Summary of baseline, hemodynamic, and hormonal data in each group of rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>MNS</th>
<th>MHS</th>
</tr>
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<tbody>
<tr>
<td>Body weight, g</td>
<td>71.0±2.6</td>
<td>72.0±2.1</td>
</tr>
<tr>
<td>Kidney weight, g</td>
<td>0.77±0.06</td>
<td>0.62±0.04*</td>
</tr>
<tr>
<td>GFR, ml/min/g</td>
<td>1.20±0.07</td>
<td>1.38±0.14*</td>
</tr>
<tr>
<td>MRB, mmHg</td>
<td>99.4±3.0</td>
<td>97.4±1.4</td>
</tr>
<tr>
<td>P&lt;sub&gt;OMS&lt;/sub&gt;, mosmol/kgH2O</td>
<td>298.3±2.3</td>
<td>293.7±1.5</td>
</tr>
<tr>
<td>Plasma AVP, pg/ml</td>
<td>9.9±1.9</td>
<td>10.4±3.2</td>
</tr>
<tr>
<td>Aldosterone, ng/dl</td>
<td>11.8±4.4</td>
<td>15.0±3.0</td>
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</table>

Values are means ± SE; n = 11 rats/group. MNS, Milan normotensive rats; MHS, Milan hypertensive rats; MBP, mean blood pressure; GFR, glomerular filtration rate; P<sub>OMS</sub>, plasma osmolality; AVP, arginine vasopressin. *P < 0.01 vs. MNS rats.
Using this technique, we examined whether NHE3 mRNA was different in the two strains of MNS and MHS rats. The quantification was performed on proximal tubules, isolated as described in METHODS; total RNA was purified simultaneously from MNS and MHS renal tissue, and the competitive PCR reactions were performed starting from the same amounts of total RNA (200 ng). For each experiment, MNS and MHS kidneys were studied in parallel. As shown in Fig. 1C, NHE3 mRNA abundance was not significantly different in proximal tubules of MNS rats (1.10 ± 0.22 fmol/ng total RNA, n = 7) vs. proximal tubule of MHS rats (0.89 ± 0.15 fmol/ng total RNA, n = 8).

NHE3 protein abundance was determined by Western blot analysis using a rabbit polyclonal antibody. As shown in Fig. 1D, the antibody recognized a protein with a molecular mass between 66 and 116 kDa. Densitometric analysis (Fig. 1E) demonstrates that NHE3 abundance, normalized for β-actin, was similar in the two strains (MNS: 1.12 ± 0.06, n = 7 vs. MHS: 0.90 ± 0.13, n = 7), thus confirming, at the protein level, the data of the competitive PCR.

NHE3 mRNA abundance in TAL was again similar in MNS (2.36 ± 0.31 fmol/ng total RNA, n = 8) to MHS (1.94 ± 0.31 fmol/ng total RNA, n = 8). The same was found for NHE3 protein abundance that, normalized for β-actin, was 1.31 ± 0.29 in MNS (n = 5) and 1.26 ± 0.21 in MHS animals (n = 5).

NKCC2 mRNA and protein abundance along the TAL. As shown in Fig. 2C, NKCC2 mRNA abundance was increased 2.4-fold in TAL of MHS rats (2.37 ± 0.24 fmol/ng total RNA, n = 8) vs. in TAL of MNS (1.00 ± 0.11 fmol/ng total RNA, n = 8).

NKCC2 protein abundance, as determined by Western blot analysis using a rabbit polyclonal antibody (Fig. 2, bottom), was again significantly (P < 0.005) increased in TAL of MHS animals (0.76 ± 0.05, n = 5) vs. in TAL of MNS rats (0.36 ± 0.03, n = 4).

NCC mRNA and protein abundance along the distal convoluted tubule. The results of the competitive PCR reactions are shown in Fig. 3, top; NCC mRNA abundance was significantly (P < 0.005) lower in distal tubules of MHS rats (0.16 ± 0.07 fmol/ng total RNA, n = 7) than in distal tubules of MNS rats (0.69 ± 0.14 fmol/ng total RNA, n = 5).

Western blot analysis performed on slices from the renal cortex (Fig. 3D) shows that NCC antibody recognized a protein with a molecular mass between 116 and 205 kDa. As illustrated in Fig. 3E, densitometric analysis demonstrated that NCC abundance, normalized for β-actin, was significantly (P < 0.01) lower in MHS rats (0.99 ± 0.07, n = 5) than in MNS animals (2.30 ± 0.44, n = 5).

α-ENaC mRNA in collecting ducts. The results of the competitive PCR reactions are shown in Fig. 4: α-ENaC mRNA abundance was significantly (P < 0.05) lower in the cortical collecting duct of MHS rats (1.38 ± 0.06 amol/ng total RNA, n = 5) compared with MNS rats (1.67 ± 0.11 amol/ng total RNA, n = 4) (Fig. 4C). The same holds for mRNA along the
outer medullary collecting ducts (Fig. 4D); it was 1.18 ± 0.02 amol/ng total RNA, n = 4, in MHS rats and 1.71 ± 0.19 amol/ng total RNA, n = 4, in MNS animals (P < 0.05).

ENaC subunit protein abundances in collecting ducts. Western blot analysis performed on slices from renal cortex (Fig. 5, top) show that α-, β-, and γ-ENaC antibodies recognized a protein with a molecular mass between 66 and 116 kDa. As illustrated in Fig. 5A, densitometric analysis demonstrated that α-ENaC abundance, normalized for β-actin, was significantly (P < 0.01) lower in MHS rats (0.68 ± 0.03, n = 5) than in MNS animals (0.77 ± 0.02, n = 5). In contrast, β-ENaC remained unchanged (0.75 ± 0.02, n = 5, in MNS and 0.76 ± 0.01, n = 5 in MHS) (Fig. 5B). The same holds for γ-ENaC (1.05 ± 0.01, n = 5, in MNS and 1.06 ± 0.01, n = 5, in MHS) (Fig. 5C).

Similar results were obtained in the inner stripe of outer medulla (Fig. 6), where α-ENaC abundance was 0.75 ± 0.02 (n = 5) in MHS compared with 0.94 ± 0.13 (n = 5) in MNS animals (P < 0.001) (Fig. 6A); β-ENaC was 1.31 ± 0.02 (n = 5) in MNS vs. 1.32 ± 0.01 in MHS (n = 5) (Fig. 6B); and γ-ENaC was 1.67 ± 0.01 (n = 5) in MNS vs. 1.64 ± 0.02 (n = 5) in MHS (Fig. 6C).

**DISCUSSION**

In the present study, we have investigated whether a modulation of the gene and protein expression of luminal sodium carriers may be involved in the development of hypertension in the genetically Milan hypertensive rats. First of all, using the inulin clearance technique, we could confirm that at this age (23–25 days) the MHS animals show an increased GFR (relative to kidney weight) but still have normal blood pressure, thus confirming earlier results (5).

**NHE3 mRNA and protein abundance in proximal tubules.** Determination of NHE3 mRNA and protein abundance in renal proximal tubules isolated from 23- to 25-day-old MHS and age-matched MNS rats reveals that, in the prehypertensive phase, NHE3 transcript and protein abundances are not significantly different in the two strains of rats, thus indicating that the modulation of gene expression does not seem to be involved in the induction of hypertension in the MHS animals. These results are in seeming contrast to a previous report demonstrating that Na⁺/H⁺ exchanger activity is increased in MHS compared with MNS rats (21). Since in the present study we have actually measured the expression and the abundance, but not the activity, of NHE3, it cannot be excluded that the contribution of the NHE3 gene to MHS hypertension is mediated by its increased activity and not necessarily associated with an overexpression. Moreover, although NHE3 has been identified as the most abundant isoform of the Na⁺/H⁺ exchanger present in the apical membrane of the proximal tubule and involved in trans-epithelial sodium reabsorption, several reports indicate that other isoforms of the Na⁺/H⁺ exchanger family, some of which are yet unidentified, may be involved in sodium transport (46). It should be stressed that the role and, more importantly, the mechanism involving NHE3 in the pathogenesis of genetic hypertension are still elusive. For example, in studies in spontaneously hypertensive rats (SHR) there are reports that indicate that NHE3 is regulated at the
Fig. 3. Sodium-chloride cotransporter (NCC) mRNA and protein abundance in 23- to 25-day-old MNS vs. MHS rats. A: ethidium bromide-stained gel for competitive PCR analysis. Shown are 453-bp internal standard cDNA and 550-bp wild-type cDNA for NCC. B: log-log plot of the ratio of quantitative fluorescence data vs. initial amount of internal standard for NCC. C: NCC mRNA abundance in distal convoluted tubules. ***P < 0.005. D: Western blot analysis results. E: protein abundance for NCC in renal cortex. **P < 0.01.

Fig. 4. α-Subunit of epithelial sodium channel (ENaC) mRNA abundance in cortical and outer medullary collecting ducts of 23- to 25-day-old MNS and MHS rats. A: ethidium bromide-stained gel for competitive PCR analysis. Shown are 605-bp internal standard cDNA and 678-bp wild-type cDNA for α-ENaC. B: log-log plot of the ratio of quantitative fluorescence data vs. initial amount of internal standard for α-ENaC. C: α-ENaC mRNA abundance in cortical (C) and outer medullary collecting duct (D). *P < 0.05.
transcriptional level (26), whereas others suggest that a post-
transcriptional mechanism is responsible for the overexpres-
sion of NHE3 protein and the associated increase in Na\(^+\)/H\(^+\) exchanger activity (27). Finally, the redistribution of NHE3 in
proximal tubule cells may be an additionally important factor
in understanding sodium transport along the proximal tubule
during acute and chronic hypertension (49).

NKCC2 mRNA and protein abundance along the TAL. One
of the new findings of the present study is the identification of
NKCC2 as a potential player in the induction of this form of
genetic hypertension. Indeed, as shown in Fig. 2, NKCC2 gene
expression, measured in the medullary TAL, is significantly
enhanced at both the mRNA and protein level in MHS com-
pared with MNS rats. It is also noteworthy to underline that the
2.4-fold increase in mRNA is not substantially different from
the 2.1-fold increase in NKCC2 protein amount, even though
this last value was corrected for \(\beta\)-actin abundance. These
results are in excellent agreement with the previous report from
Ferrandi et al. (15) demonstrating that NKCC2 activity (mea-
sured as bumetanide-sensitive \(^86\)Rb uptake) in membrane ves-
cicles isolated from the same zone of the kidney, i.e., the outer
medulla, is significantly increased in MHS compared with
MNS rats (15). Moreover, they fit very well with another report
from the same group demonstrating a major rise in the abun-
dance and activity of the Na\(^+\)-K\(^+\)-ATPase in the outer medulla
of MHS animals (16).

The strong stimulation of NKCC2 could potentially contrib-
ute to the significant rise in blood pressure. Several lines of
evidence support this hypothesis. First of all, the TAL is an
important site of sodium transport; actually, roughly 20% of
the filtered sodium load is normally reabsorbed at this level.
Second, apical sodium entry in the TAL is mainly mediated by
NKCC2 (18) as demonstrated by the copious natriuresis asso-
ciated with the use of loop diuretics, an effect that is enhanced
by their inhibiting action on TGF (48). Third, patients carrying
a mutation with a loss of function of the NKCC2 gene (type I
Bartter’s syndrome) are characterized by orthostatic hypoten-
sion (8). Finally, mice lacking the NKCC2 gene suffer from
severe salt wasting resulting in rapid death (44). It is noteworthy
to underline that upregulation of NKCC2 has been also
reported in the early phase of prenatally programmed hyper-
tension induced by a maternal low-protein diet during preg-
nancy (30).

The stimulation of NKCC2 may also be involved in the
increased GFR found in the MHS animals. In fact, activation of
ion transport along the TAL is expected to decrease the
delivery of sodium chloride to the macula densa, thus enhanc-
GFR through the tubuloglomerular feedback mechanism.
In young MHS rats, the alteration of TGF, together with an
increase in net interstitial pressure, has already been described
by Boberg and Persson (6), whereas in the present paper we
have probably identified the molecular mechanism involved in
this process.

NCC mRNA and protein abundance in the distal convoluted
tubule. Our findings further indicate that NCC gene transcript
and protein abundances along the distal convoluted tubule are

Fig. 5. \(\alpha\) (A), \(\beta\) (B), and \(\gamma\)-ENaC (C) protein abundance in renal cortex of kidneys from 23- to 25-day-old MNS and MHS animals as detected by Western
blot analysis. **P < 0.01.
significantly decreased in MHS rats compared with MNS (Fig. 3). This effect may be related to decreased sodium load that reaches the distal tubule due to the larger trans-epithelial sodium transport along the TAL linked to the overexpression of NKCC2. Indeed, it has been demonstrated that high rates of ion delivery to the distal nephron, following chronic administration of furosemide, raised [3H]metolazone binding sites (an index of NCC function) in membranes extracted from renal cortex (11) and enhanced NCC transcript levels in distal tubular cells (35). Although in the classic “escape” phenomenon either blood pressure is raised or aldosterone levels are clamped high, it is possible that the decrease in NCC abundance may be related to the “escape” phenomenon, as it has been demonstrated in animal models (47). Such a hypothesis is supported by three observations: first, the unchanged aldosterone plasma levels indicate that this phenomenon is independent from aldosterone-mediated gene regulation; second, at this age, the MHS rats are in a state of a slight volume expansion (4) and the decrease in the abundance of NCC may be necessary to restore sodium balance; and third, the young MHS rats exhibited a significantly higher net interstitial pressure than the age-matched MNS (6). This last finding may indicate that NCC is the key molecular target of interstitial pressure variations as it has been suggested in animal models of long-term regulation of pressure natriuresis (29).

ENaC subunits in the collecting ducts. In the present study, we have also examined the expression of ENaC that is the key apical Na⁺ transporter from the connecting tubule to the collecting duct. ENaC is a heteromultimeric channel, composed of three homologous α-, β-, and γ-subunits (9). It has been shown that α-ENaC is mostly present at the apical domains of the principal cells, whereas β- and γ-ENaC are mainly localized in cytosolic vesicles (20). The significance of this different subcellular distribution of the three subunits has not been ascertained. Oocyte injection experiments demonstrate that the α-subunit is essential for the function of the channel but that the channel activity is enhanced by association with β- and γ-subunits (2), a finding that has been confirmed by the generation of gene knockout mice for the individual subunits (23). Finally, aldosterone and vasopressin regulate ENaC-dependent sodium reabsorption by changing the expression of the individual ENaC subunits (14, 32). In accordance with the results obtained for NCC, our present findings indicate

![Fig. 6. α (A)-, β (B)-, and γ-ENaC (C) protein abundance in the inner stripe of outer medulla of kidneys of kidneys from 23- to 25-day-old MNS and MHS animals as detected by Western blot analysis. ***p < 0.005.](http://ajprenal.physiology.org/)
that both in the cortex and in outer medulla of MHS kidneys there is a modest, although significant, downregulation of α-ENaC. Since apical Na\(^+\) entry is believed to constitute the rate-limiting step in Na\(^+\) reabsorption in the different nephron segments, it is likely that decreased expression of apical membrane Na\(^+\) carriers reflects inhibition of transepithelial Na\(^+\) transport. It is therefore obvious to postulate that in this model of sodium-dependent hypertension, the early phase is characterized by decreased Na\(^+\) transport in the tubular segments beyond the TAL. Such a phenomenon is not unique to this model, because in the prenatally programmed hypertension the vigorous upregulation of NKCC2 is partially compensated for by a slight reduction of α-ENaC (30). With respect to the β- and γ-subunits, the lack of changes is not surprising because, in other experimental models associated with changes in renal sodium handling, the regulation of the channel may be dependent on other factors like a degradation of the γ-subunit (43). We must emphasize that with our technical approach we are able to detect only changes in channel protein abundance, whereas epithelial sodium reabsorption via ENaC may be controlled in addition by changing the conductance or the gating kinetics of functional channels.

**Cellular and hormonal mediators.** The important finding of our study is the increased NKCC2 gene expression in MHS rats. Several hypotheses may be advanced to identify the signaling mechanism(s) responsible for this effect. The first possible mechanism is superoxide. Recently, it has been demonstrated that the TAL is the major source of superoxide production (28); in addition, superoxidestimulates sodium chloride absorption along the TAL (36), and our preliminary data indicate that the kidney of MHS generates more superoxide compared with MNS animals (Capasso G, Cantone A, and Poulet R, unpublished observations). All these studies may indicate a role of superoxide in the modulation of the function of NKCC2. Second, it could be related to morphological changes in the kidney and tubular cells; in fact, it has been demonstrated that the size of the kidneys and the volume of the tubular cells in MHS are supposed to be smaller than MNS rats (17). This would necessitate a higher GFR-to-kidney size ratio to filter the blood; it is therefore reasonable to postulate that the increased single-nephron sodium load may trigger the reported upregulation of TAL sodium reabsorption. These findings are quite intriguing, because in rats with low birth weight induced by a maternal low-protein diet, the development of hypertension has been related to transcriptional upregulation of NKCC2 (30). Finally, it is still possible that enhanced expression of NKCC2 in MHS may be dependent on aldosterone and/or vasopressin (ADH). However, we have not found any statistical difference in aldosterone levels between MNS and MHS, thus confirming previous results from Mantero et al. (31); the same holds for ADH plasma levels between the two strains of rats (Table 2). Although measurements of these hormones in young rats are extremely difficult and the results are largely variable, it looks as if aldosterone and ADH may not be the possible causes of the increased expression of NKCC2.

**Conclusions.** We have found that in young MHS animals, characterized by increased GFR and a still normal blood pressure, the expression and abundance of NHE3, measured along the proximal tubule and the TAL, are not different, whereas the NKCC2 transporter, expressed along the medullary TAL, is upregulated compared with in age-matched MNS animals. Along the distal convoluted tubule, NCC gene transcript and protein abundances are significantly reduced in MHS. Finally, in the collecting ducts there is downregulation of gene expression and protein abundance of α-ENaC. Therefore, the upregulation of NKCC2 along the medullary TAL does not induce an increase in systemic blood pressure, probably because its action on salt reabsorption is offset by the downregulation of NCC and α-ENaC, and by the concomitant increase in GFR.

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