Progression of microalbuminuria in SHR is associated with lower expression of critical components of the apical endocytic machinery in the renal proximal tubule

Heart Institute (InCor), University of São Paulo Medical School, São Paulo, Brazil
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Inoue BH, Arruda-Junior DF, Campos LC, Barreto AL, Rodrigues MV, Krieger JE, Girardi AC. Progression of microalbuminuria in SHR is associated with lower expression of critical components of the apical endocytic machinery in the renal proximal tubule. Am J Physiol Renal Physiol 305: F216–F226, 2013.—Cumulative epidemiological evidence indicates that the presence of microalbuminuria predicts a higher frequency of cardiovascular events, peripheral disease, and mortality in essential hypertension. Microalbuminuria may arise from increased glomerular permeability and/or reduced proximal tubular reabsorption of albumin by receptor-mediated endocytosis. This study aimed to evaluate the temporal pattern of urinary protein excretion and to test the hypothesis that progression of microalbuminuria is associated with decreased protein expression of critical components of the endocytic apparatus in the renal proximal tubule of spontaneously hypertensive rats (SHR). We found that urinary albumin excretion increased progressively with blood pressure in SHR from 6 to 21 wk of age. In addition, SDS-PAGE analysis of urinary proteins showed that microalbuminuric SHR virtually excluded proteins of the size of albumin or smaller (<70 kDa), typical of tubular proteinuria. Moreover, the protein abundance of the endocytic receptors megalin and cubilin as well as of the chloride channel ClC-5 progressively decreased in the renal cortex of SHR from 6 to 21 wk of age. Expression of the vacuolar H^+-ATPase B2 subunit was also reduced in the renal cortex of 21-wk-old compared with both 6- and 14-wk-old SHR. Collectively, our study suggests that enhanced urinary protein excretion, especially of albumin, may be due, at least in part, to lower expression of key components of the apical endocytic apparatus in the renal proximal tubule. Finally, one may speculate that dysfunction of the apical endocytic pathway in the renal proximal tubule may contribute to the development of microalbuminuria in essential hypertension.

receptor-mediated endocytosis; megalin; cubilin; ClC-5; vacuolar H^+-ATPase; hypertension

The identification and quantitation of proteinuria, particularly of albumin, are of great importance in the initial diagnosis and at subsequent follow-up in kidney disease patients (40, 48, 60). Massive and sustained proteinuria is an unequivocal sign of established nephropathy while detection of small increases in the normal amount of urinary albumin excretion, known as microalbuminuria (30–300 µg/24 h or 20–200 µg/min) (40, 60), is indicative of renal dysfunction and renal disease progression. Increased urinary albumin excretion results from enhanced glomerular filtration and/or decreased reabsorption of this macromolecule in the renal proximal tubule (14). Under physiological conditions, about 170 mg·9 g of albumin are filtered daily by human glomeruli, but <30 mg are indeed excreted in the urine largely due to protein reabsorption in the renal proximal tubule via clathrin-dependent, receptor-mediated endocytosis, an essential mechanism for the transport of macromolecules into the cells as well as across the epithelia (8, 42, 57). Receptor-mediated endocytosis in the apical membrane of the proximal tubule involves binding of albumin, among a number of other ligands, to the receptor megalin and to its intracellular binding partner cubilin (5, 17, 47). Once the ligand-receptor complex is internalized, it is directed to the endosomal compartment where the complex is dissociated; the receptors megalin/cubilin recycle back to the apical plasma membrane whereas albumin is degraded in the lysosomes to its constituent amino acids that are released into the bloodstream (13, 17).

In addition to megalin and cubilin, the endocytosis of albumin requires the coordinated action of a macromolecular complex, which includes the Na^+/H^+ exchanger isoform 3 (NHE3), the vacuolar proton pump (v-H^+-ATPase), and the chloride channel ClC-5 (17, 22, 24, 26, 32, 36, 47, 53). These transport proteins are mainly involved in the maintenance of pH homeostasis of endosomal compartments, which is essential to the endocytic process, and influences not only the ligand-receptor dissociation but also the vesicle trafficking, endosomal fusion events, and recycling of receptors to the plasma membrane (20, 38, 42, 54). The role of megalin, cubilin, NHE3, ClC-5, and v-H^+-ATPase as critical components of the proximal tubule endocytic machinery is supported by several studies conducted in knockout mice (22, 24, 46, 62). These experimental animal models (6, 52, 58) as well as a number of diseases characterized by tubular proteinuria have been shown to be associated with decreased renal expression of these receptors and/or transporters (2, 32, 44, 56).

Microalbuminuria is an independent risk factor for cardiovascular disease and renal disease progression (4, 11, 37, 40, 49). In essential hypertension, glomerular endothelial dysfunction, intraglomerular hypertension, and hemodynamic maladjustment as well as podocyte injury are believed to contribute to microalbuminuria (11, 16, 41, 49). Alternatively, Russo and colleagues (50) have found that the presence of microalbuminuria in spontaneously hypertensive rats (SHR) is independent of changes in glomerular permeability, suggesting that defective uptake of filtered albumin by the renal proximal tubule may represent an important mechanism underlying the development of microalbuminuria in this experimental model. The present study was...
Table 1. Primers used for real-time RT-PCR and predicted amplicon size

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Lrp2, megalin receptor; cubn, cubilin receptor; Atp6v1b2, Clcn5, chloride channel 5 (ClC-5); v-H + ATPase transporter B2 subunit; Nphs1, nephrin; Nphs2, podocin; Ppi1, peptidylprolyl isomerase A (cyclophilin A); S, sense; AS, antisense.

design to evaluate the temporal pattern of urinary protein excretion and to test the hypothesis that progression of microalbuminuria is associated with lower renal expression of critical components of the endocytic machinery that mediate the reabsorption of albumin in the renal proximal tubule of spontaneously hypertensive rats.

MATERIALS AND METHODS

Materials. A monoclonal antibody directed to megalin was a kind gift from Dr. Daniel Biemesderfer (Yale University School of Medicine, New Haven, CT). We purchased polyclonal antibodies to transferrin, podocin, and nephrin, and a monoclonal antibody to v-ATPase B2 subunit from Santa Cruz Biotechnology (Santa Cruz, CA), a polyclonal antibody to ClC-5 from Alpha Diagnostic International (San Antonio, TX), and a monoclonal antibody to actin from Merck (Darmstadt, Germany). Horseradish peroxidase-conjugated goat anti-mouse, goat anti-rabbit, and rabbit anti-goat secondary antibodies were purchased from Life Technologies (Carlsbad, CA). All other reagents and chemicals were acquired from Sigma-Aldrich (St. Louis, MO), unless otherwise specified.

Experimental animals. All procedures were carried out in accordance with the ethical principles of animal research of the Brazilian College of Animal Experimentation and were approved by the Institutional Animal Care and Use Committee. Studies were performed in male SHR and age-matched Wistar rats. Rats were housed at the University of São Paulo Medical School animal facility under standardized conditions (constant temperature of 22°C, 12:12-h light-dark cycle, and relative humidity of 60%). Blood pressure and renal function evaluation were performed at 6, 14, and 21 wk of age. Arterial blood was collected from the right ventricle at the time of death for measurements of serum creatinine levels. Kidneys were removed, weighted, and used for preparation of renal cortical membranes or RNA extraction immediately after the rats had been euthanized by decapitation.

Blood pressure measurement. Blood pressure was measured by tail-cuff plethysmography (BP-2000 Blood Pressure Analysis System, Visitech Systems, Apex, NC). Before these measurements, rats were trained in the blood pressure device to become adapted to the experimental procedures.

Renal function evaluation. Rats were individually placed into metabolic cages during 4 consecutive days: the first day to adapt the rats to the cages and the following days to assess urine function. Urine samples collected during each 24-h period were used to determine urine output, glomerular filtration rate (GFR), urinary creatinine, and sodium and protein excretion. Urine output was measured gravimetrically. Creatinine clearance was used to estimate GFR. Serum and urinary creatinine concentrations were measured by a kinetic method (Labtest, Minas Gerais, Brazil) using a Thermoplate Analyzer Plus (ThermoPlate, São Paulo, Brazil). Sodium was measured on a Radiometer ABL800 Flex (Radiometer Medical, Brønshøj, Denmark). Urinary protein excretion was determined using a Sensiprot kit (Labtest).

Urine albumin excretion. Urinary albumin concentration was determined with an ELISA kit specific for rat urine albumin (Nephrat kit; Exocell, Philadelphia, PA). Experiments were carried out following the manufacturer’s instructions. Additionally, albumin was isolated from urine by SDS-PAGE and quantified by densitometry using ImageJ Software (Scion, Frederick, MD).

Preparation of renal cortical membranes. Rat renal cortices were separated at 4°C, minced with opposing razor blades, and homogenized in a Potter-Elvehjem-style tissue grinder (Polymix PX-SR 50; Kinematics, Bohemia, NY) for 25 strokes in ice-cold PBS (10 mM phosphate, 140 mM NaCl, pH 7.4) containing protease (1 mM pepstatin, 1 mM leupeptin, and 230 mM PMSF) and phosphatase inhibitors (15 mM NaF and 50 mM sodium pyrophosphate). Renal cortical membranes were prepared by differential centrifugation of renal cortical homogenates from individual animals as described previously (12). Protein concentration was measured by the method of Lowry (33).

SDS-PAGE. Equivalent protein amounts of renal cortical membranes or a volume of urine containing 5 or 40 μg of creatinine were solubilized in SDS sample buffer (2% SDS, 10% glycerol, 0.1% bromophenol blue, 50 mM Tris, pH 6.8), and proteins were separated by SDS-PAGE using 7.5 or 10% polyacrylamide gels.
according to Laemmli (31). Following electrophoresis, some gels were silver stained using a ProteoSilver Plus kit to detect urinary proteins.

**Immunoblotting.** Renal cortical membrane proteins were transferred to polyvinylidene difluoride membranes (PVDF; Immobilon-P, Millipore) from polyacrylamide gels at 500 mA for 5 h at 4°C with a TE 62 Transfer Cooled Unit (GE HealthCare, Piscataway, NJ) and stained with Ponceau S in 0.5% trichloroacetic acid. PVDF membranes containing transferred proteins were incubated first in blotto (5% nonfat dry milk and 0.1% Tween 20 in PBS, pH 7.4) for 1 h, followed by overnight incubation in primary antibody. Primary antibodies were diluted in blotto in concentrations ranging from 1:500 to 1:50,000. The membranes were then washed five times in blotto and incubated for 1 h with horseradish peroxidase-conjugated immunoglobulin secondary antibody (1:2,000). Bound antibody was detected using an enhanced chemiluminescence system (GE Healthcare) according to the manufacturer’s protocols. The signals were captured using an ImageQuant LAS 4000 mini (GE HealthCare) and quantified using ImageJ Software (Scion).

**RNA extraction and real-time RT-PCR.** Total RNA was isolated from rat renal cortices by using TRIzol Reagent (Life Technologies) according to the manufacturer’s specifications. First-strand cDNA was synthesized using a random High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA) following the manufacturer’s guidelines. Quantitative real-time RT-PCRs were carried out using SYBR Green PCR Master Mix-PE (Applied Biosystems) on the ABI Prism 7700 Sequence Detection System (Applied Biosystems). The oligonucleotide primers used to detect megalin, cubilin, ClC-5, v-H+/H11001-ATPase B2-subunit, nephrin, podocin, and the internal control cyclophilin are shown in Table 1. mRNA analyses of target genes were

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**Fig. 2. Evaluation of blood pressure and renal function in SHR and Wistar rats. A:** SBP in SHR and Wistar rats was measured by plethysmography. **B-D:** rats were placed in metabolic cages for 24-h urine collection for a period of 3 consecutive days. Creatinine clearance was used to estimate glomerular filtration rate (GFR). **E:** urinary protein excretion (mg/24 h) was measured using a commercially available kit based on the pyrogallol red-molybdate method. **F:** urine protein-to-creatinine ratio. Values are means ± SE; n = 19 rats/group, except for 14-wk-old SHR (n = 25) and 21-wk-old SHR (n = 20). ***P < 0.01 and ****P < 0.001 vs. 6-wk-old Wistar rats. ***P < 0.01 and ****P < 0.001 vs. 6-wk-old SHR.
assayed in triplicate. The comparative threshold (CT) cycle method was used for data analyses.

**Histological and morphometric analysis.** The kidneys from 6–8 rats/group were perfused with PBS and with 4% paraformaldehyde in PBS via the left ventricle. The kidneys were removed, weighed, fixed overnight in 4% paraformaldehyde in PBS, and embedded in paraffin. For morphometric analysis, 3-μm-thick sections were stained with hematoxylin-eosin (HE). Renal sections were subsequently examined under light microscopy, and the images and measurements were acquired using a Leica DM2700 M microscope and Leica Qwin software (version 2.2), respectively. Twenty glomeruli per slide were chosen for analysis. The major (D) and minor (d) diameters and area of glomeruli and Bowman’s capsule were measured. Determination of the mean glomerular diameter (M) and volume (V) of glomeruli and Bowman’s capsule was calculated using the following equations: 

\[ M = \sqrt{D \times d} \]

\[ V = \pi/6 \times M^3 \]

The formula

\[ A = \pi/4 \times M^2 \]

was used to confirm the area measured.

**Statistical analysis.** Data are expressed as means ± SE, with n indicating the number of observations. Comparisons among groups were made by one-way ANOVA, unless indicated otherwise. Adjustment for multiple comparisons was made with the Bonferroni method. Differences were considered significant if \( P < 0.05 \).

**RESULTS**

**Correlation between urinary protein excretion and blood pressure in SHR.** Regression analysis was performed to examine whether urinary protein excretion was correlated with systolic blood pressure (SBP) at early ages in SHR. To this end, we plotted the values of SBP against the urinary protein/creatinine ratio from 25 SHR ranging from 6 to 21 wk of age. As shown in Fig. 1, urinary protein excretion increases significantly with SBP in SHR during the analyzed period (Spearman correlation coefficient, \( r = 0.9244; P < 0.0001 \) (Fig. 1A)). Correlation between these variables was not observed in age-matched normotensive Wistar rats (\( r = 0.3147, P = 0.11 \)) (Fig. 1B). To evaluate the pattern of urinary protein excretion and to test the hypothesis that progression of proteinuria is associated with diminished expression of critical components of the apical endocytic apparatus in the renal proximal tubule of SHR, all the following experiments were undertaken in 6-, 14-, and 21-wk-old SHR. Age-matched Wistar rats served as the reference age control.

**Evaluation of blood pressure and renal function in SHR and Wistar rats.** The temporal evaluation of blood pressure and renal function in 6-, 14-, and 21-wk-old SHR and Wistar rats of the corresponding age is illustrated in Fig. 2. Figure 2A shows that SBP values increased from 6 to 14 wk of age by 10.220.32.247 on June 24, 2017 http://ajprenal.physiology.org/ Downloaded from
(110 ± 3 vs. 180 ± 2 mmHg, \( P < 0.001 \)) and continued to rise from 14 to 21 wk of age in SHR (178 ± 3 vs. 198 ± 2 mmHg, \( P < 0.001 \)). In contrast, a small but significant increase in blood pressure was observed from 6 to 14 wk of age in Wistar rats (105 ± 1 vs. 120 ± 1 mmHg, \( P < 0.001 \)), but no additional increment was observed from 14 to 21 wk of age in the lineage of normotensive animals [120 ± 1 vs. 123 ± 2 mmHg, not significant (NS)].

Fig. 5. Analysis of megalin, cubilin, ClC-5, and v-H^+-ATPase B2-subunit protein expression in renal cortex of SHR and Wistar rats. A: megalin. B: cubilin. C: chloride channel ClC-5. D: v-H^+-ATPase B2 subunit. Top: equivalent samples (5 μg for megalin, cubilin and actin, 30 μg for v-H^+-ATPase B2 subunit, and 50 μg for ClC-5) of renal cortical membranes isolated from 6-, 14-, and 21-wk-old SHR and Wistar rats were subjected to SDS-PAGE, transferred to PVDF membranes, and analyzed by immunoblotting. Bottom: graphical representation of the relative protein expression levels normalized to actin. Values are means ± SE; \( n = 8 \) rats/group. *\( P < 0.05 \), **\( P < 0.01 \), and ***\( P < 0.001 \) vs. 6-wk-old Wistar rats. **\( P < 0.01 \) and ###\( P < 0.001 \) vs. 6-wk-old SHR.
The patterns of variation of urine output, urinary sodium excretion, and GFR were similar between the two rat strains. Urine output (Fig. 2B), urinary sodium excretion (Fig. 2C), and GFR (Fig. 2D) were higher in 6-wk-old animals compared with 14- and 21-wk-old rats of the same lineage. These renal function parameters remain unchanged from 14 to 21 wk of age in both SHR and Wistar rats.

Consistent with the findings shown in Fig. 1, daily urinary excretion of protein progressively increased in SHR throughout the experimental period (Fig. 2E). A significant increase in the daily urinary excretion of protein was noted between 6- and 14-wk-old Wistar rats, but no further changes were observed between 14- and 21-wk-old normotensive animals (Fig. 2E). To minimize the differences in GFR and urinary losses that may result in underestimated values of protein excretion; we normalized protein excretion by creatinine (Fig. 2F). As expected, the urinary protein-to-creatinine ratio progressively increased in SHR (0.51 ± 0.04; 2.32 ± 0.09; and 2.93 ± 0.22 at 6, 14, and 21 wk of age, respectively), whereas the ratio increased from 6 to 14 wk (0.47 ± 0.05 vs. 0.61 ± 0.06, P < 0.05) and remained unchanged between 14- and 21-wk-old Wistar rats (0.84 ± 0.07 vs. 0.92 ± 0.08, NS).

**Evaluation of albumin excretion in SHR and Wistar rats.** Urinary albumin excretion was quantified by ELISA (Fig. 3A) and analyzed by SDS-PAGE (Fig. 3B). SHR exhibited a progressive increase in urinary albumin excretion from 6 to 21 wk of age (Fig. 3A), while albumin excretion remained within the normal range (<30 mg/day) in normotensive Wistar rats and in 6-wk-old SHR.

The profile of urinary proteins excreted by SHR and Wistar animals was evaluated by SDS-PAGE, and the amount of intact albumin was semiquantitatively determined by densitometry (Fig. 3B). The results from these experiments were very similar to the ones obtained in Fig. 3A. As seen in the bottom panel of Fig. 3B, there was a continuing increase in the levels of intact albumin in the urine of Wistar rats throughout the experimental period (94 ± 6, 2,318 ± 250, and 3,778 ± 665 in 6-, 14-, and 21-wk-old Wistar rats, respectively), but no statistical difference was observed between 14- and 21-wk-old normotensive animals (P > 0.05). The increase in the urinary levels of intact albumin was much more pronounced in SHR (70 ± 30, 15,456 ± 1,663, and 37,166 ± 1,692 arbitrary units in 6-, 14-, and 21-wk-old SHR, respectively). Most importantly, the molecular weights of proteins excreted in the urine of the animals displaying microalbuminuria (14- and 21-wk-old SHR) were similar in size to albumin or smaller, typical of tubular proteinuria (Fig. 3B).

**Evaluation of transferrin excretion in SHR and Wistar rats.** The qualitative change in the levels of urinary transferrin, a protein that is known to be reabsorbed from the glomerular ultrafiltrate by receptor-mediated endocytosis (30), was also evaluated in 24-h urine samples from SHR and Wistar rats. Figure 4 shows the results of immunoblot analyses of transferrin in the urine of these experimental animal models. Virtually no excretion of transferrin could be observed in 24 urine samples obtained from normotensive Wistar rats and from 6-wk-SHR (n = 4 animals/group) while transferrin content progressively increased in the urine of 14- and 21-wk-old SHR.

**Evaluation of protein and mRNA expression of megalin, cubilin, ClC-5, and the v-ATPase B2 subunit in the renal cortex of SHR and Wistar rats.** The results obtained above suggest tubular proteinuria in SHR, so we next evaluated whether

**Fig. 6. Analysis of megalin, cubilin, ClC-5, and V-H+ -ATPase B2-subunit mRNA expression in renal cortex of SHR and Wistar rats.** The levels of mRNA of selected key components of the endocytic machinery were measured by real-time PCR, and cyclophilin was used as an internal control. Graphical representation of the relative gene expression of megalin (A), cubilin (B), chloride channel ClC-5 (C), and V-H+ -ATPase B2 subunit (D) in the renal cortex of SHR and Wistar rats is shown. Values are means ± SE. n = 6 rats/group. *P < 0.05 and **P < 0.01 vs. 6 wk-old Wistar rats. #P < 0.05, #P < 0.01 and $$P < 0.001$ vs. 6-wk-old SHR.
The progression of microalbuminuria was associated with a lower expression of selected proteins involved in the process of receptor-mediated endocytosis in renal proximal tubule of genetic hypertensive animals. Protein abundance (Fig. 5) and the mRNA levels (Fig. 6) of megalin, cubilin, CIC-5, and the v-H\(^{+}\)-ATPase B2 subunit were examined in the renal cortex of SHR and Wistar rats. Regulation of the B2 subunit of the v-H\(^{+}\)-ATPase was examined, as opposed to other subunits, because it is known that B2 is expressed in the apical membrane of the proximal tubule where it plays a role in the acidification of intracellular organelles and possibly in trans-epithelial proton secretion (45), and because we have previously found that the activity of the v-H\(^{+}\)-ATPase is regulated by changes of the B2 subunit in response to angiotensin II (9) and by both CIC-5 and CFTR in the renal proximal tubule (10).

We noticed a similar pattern for renal cortical protein abundance of megalin, cubilin, CIC-5, and the v-H\(^{+}\)-ATPase B2 subunit in normotensive Wistar rats (Fig. 5, A–D). The relative expression of these four proteins normalized to actin increased in both 14- and 21-wk-old rats compared with 6-wk-old Wistar rats. Conversely, the protein abundance of megalin (Fig. 5A), cubilin (Fig. 5B), and CIC-5 (Fig. 5C) progressively decreased in the renal cortex of SHR. Reduction of megalin protein expression was \(50\%\) at 14 wk of age and \(70\%\) at 21 wk of age compared with 6-wk-old SHR. The relative protein expression levels of cubilin decayed \(40\%\) at 14 wk of age and \(50\%\) at 21 wk of age compared with 6-wk-old SHR. Similarly, the relative protein levels of CIC-5 were \(60\%\) lower at 14 wk of age and \(80\%\) lower at 21 wk of age compared with 6-wk-old SHR. Regarding cortical expression of the v-H\(^{+}\)-ATPase B2 subunit, no statistical difference was observed between 6-wk-old and 14-wk-old SHR; however, a significant decrease was observed in 21-wk-old SHR (\(P < 0.001\) and \(29 \pm 3\%\) vs. 14 wk-old SHR, \(P < 0.05\)) (Fig. 5D).

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**Fig. 7.** Analysis of nephrin and podocin protein abundance and mRNA expression in renal cortex of SHR and Wistar rats. A: equivalent samples (20 \(\mu\)g for nephrin and 30 \(\mu\)g for podocin) of renal cortical membranes isolated from 6-, 14-, and 21-wk-old SHR and Wistar rats were subjected to SDS-PAGE, transferred to PVDF membranes, and analyzed by immunoblotting. Graphical representation of the relative protein expression levels of nephrin (B) and podocin (C) normalized to actin as well as of the relative gene expression of nephrin (D) and podocin (E) in the renal cortex of SHR and Wistar rats are shown. mRNA expression was measured by real-time PCR, and cyclophilin was used as an internal control. Values are means ± SE; \(n = 6–8\) rats/group. *\(P < 0.05\), **\(P < 0.01\) and ***\(P < 0.001\) vs. 6-wk-old Wistar rats. *\(P < 0.05\), **\(P < 0.01\), and ***\(P < 0.001\) vs. 6-wk-old SHR.
The relative mRNA expression of megalin, cubilin, CIC-5, and the v-H^+/ATPase B2 subunit in renal cortex from SHR and Wistar rats was determined by real-time RT-PCR using the specific primers depicted in Table 1. The expression of these transcripts was normalized by cyclophilin. As illustrated in Fig. 6, the pattern of mRNA expression of these four genes was consistent with the one observed at the protein level in the renal cortex of Wistar rats. Interestingly, this pattern was also observed for megalin, cubilin, and CIC-5 transcripts in the renal cortex of SHR. As opposed to the progressive decrease in protein levels (Figs. 5, A–C), the mRNA expression of megalin (Fig. 6A), cubilin (Fig. 6B), and CIC-5 (Fig. 6C) was significantly higher in both 14- and 21-wk-old rats compared with 6-wk-old SHR. On the other hand, changes in v-H^+/ATPase B2 subunit mRNA (Fig. 6D) were associated with changes in protein expression (Fig. 5D) in the renal cortex of SHR.

Evaluation of protein and mRNA expression of nephrin and podocin in the renal cortex of SHR and Wistar rats. Recent evidence suggests that the most selective barrier for the ultrafiltration of proteins resides in the glomerular slit diaphragm (14, 39, 59). Because a decrease in the expression of the slit diaphragm molecular components, nephrin and/or podocin (1, 15, 25, 29, 34), has been associated with proteinuria, we also evaluated whether nephrin and podocin expression diminishes with a blood pressure increase in SHR.

As illustrated in Fig. 7, a similar temporal expression profile for renal cortical nephrin and podocin protein and mRNA was observed in both strains. Immunoblot analysis showed that the relative protein abundance of nephrin, normalized by actin, was significantly higher in adult Wistar and SHR rats (at 14 and 21 wk of age) compared with rats of the same lineage at 6 wk of age (Fig. 7, A and B). On the other hand, the protein abundance of podocin remained unaltered with age in both SHR and Wistar rats (Fig. 7, A and C). Analysis of the relative abundance of nephrin (Fig. 7D) and podocin (Fig. 7E) transcripts in the renal cortex of SHR and Wistar rats indicates that the expression of these slit diaphragm components is significantly higher in both 14- and 21-wk-old compared with 6-wk-old rats. Interestingly, whereas Wistar rats had approximately twofold greater nephrin mRNA levels at 14 and 21 wk compared with 6 wk, nearly a fourfold increase was observed in SHR during the same experimental period (Fig. 7D).

Evaluation of renal hypertrophy in SHR. Hypertension may lead to renal hypertrophy and glomerular injury that are not always associated with altered renal hemodynamics (3, 27). We therefore evaluated whether renal hypertrophy, assessed as the kidney weight/body weight ratio, as well as glomerular volume (43), a key determinant of glomerular structural changes, were altered in SHR throughout the experimental period compared with normotensive Wistar rats.

As depicted in Table 2, body weight and kidney weight progressively increased with age in both SHR and Wistar rats. SHR displayed significantly lower body weight and kidney weight than that of age-matched Wistar rats ($P < 0.001$). The kidney weight/body weight ratio was higher in 6-wk-old Wistar rats and in SHR compared with 14- and 21-wk-old rats of the same lineage. There was no difference between SHR and age-matched Wistar rats in terms of the kidney weight/body weight ratio, suggesting that there was no renal hypertrophy in SHR during this experimental period.

The results of glomerular volume estimation are shown in Table 2. In both SHR and Wistar rats, mean glomerular volume significantly increased from 6 to 14 wk of age, but remained unchanged from 14 to 21 wk of age. Similarly, Bowman’s capsule volume was higher in 14- and 21-wk-old than in 6-yr-old rats. In concert, these findings suggest that increased urinary excretion of albumin in SHR, among other proteins, was not associated with glomerular hypertrophy during this experimental period.

DISCUSSION

The results from the present study provide evidence supporting the view that tubular dysfunction represents an important contributing mechanism underlying microalbuminuria in hypertension. First, despite the progressive increase in microalbuminuria and urinary transferrin excretion between 14- and 21-wk-old SHR, GFR and mean glomerular volume remained unchanged during this period, thus minimizing the role of glomerular hyperfiltration of proteins. Second, analyses of urine samples by SDS-PAGE showed that microalbuminuric SHR only excreted proteins with molecular weight similar or smaller than albumin (70 kDa), which resembles the profile of urinary proteins excreted by typical models of tubular proteinuria observed in megalin and CIC-5 knockout mice. Third, progression of microalbuminuria in SHR throughout the experimental period was associated with lower expression of key components of the apical endocytic machinery in the renal proximal tubule. Finally, we found no evidence for changes in the protein abundance of the glomerular proteins nephrin (28) or podocin (7) over the 21-wk experimental period.

### Table 2. Physiological and renal morphometric parameters

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<tr>
<td>Kidney wt, mg</td>
<td>2089 ± 51 (8)</td>
<td>3199 ± 121*** (8)</td>
</tr>
<tr>
<td>Kidney wt/body wt, mg/g</td>
<td>9.37 ± 0.16 (8)</td>
<td>6.94 ± 0.19** (8)</td>
</tr>
<tr>
<td>Glomerular volume, μm^3 × 10^14</td>
<td>12.7 ± 1.6 (6)</td>
<td>29.5 ± 1.0** (6)</td>
</tr>
<tr>
<td>Glomerular area, μm^2 × 10^6</td>
<td>2.9 ± 0.24 (6)</td>
<td>5.2 ± 0.11** (6)</td>
</tr>
<tr>
<td>Bowman’s capsule volume, μm^2 × 10^4</td>
<td>21.2 ± 2.5 (6)</td>
<td>50.3 ± 1.8* (6)</td>
</tr>
<tr>
<td>Bowman’s capsule area, μm^2 × 10^4</td>
<td>4.2 ± 0.30 (6)</td>
<td>7.5 ± 0.1* (6)</td>
</tr>
</tbody>
</table>

Values are means ± SE. Number of animals per group is indicated in parenthesis. SHR, spontaneously hypertensive rats. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ vs. 6-wk-old Wistar rats. #$P < 0.5$, ##$P < 0.01$, and ###$P < 0.001$ vs. 6-wk-old SHR.
Uptake of albumin by the proximal tubules from glomerular filtrate occurs via receptor-mediated endocytosis, which requires the formation of the megalin/cubilin receptor complex and several accessory plasma membrane transport proteins. We found that the protein expression of the endocytic receptors megalin and cubilin, as well as of the chloride channel CIC-5 and of the v-H^+ -ATPase B2 subunit progressively decreases in SHR from 14 to 21 wk of age, indicating that receptor-mediated endocytosis is compromised at this stage of hypertension. To our knowledge, this is the first study that shows that microalbuminuria in SHR is associated with lower renal cortical expression of megalin, cubilin, and the v-ATPase B2 subunit. On the other hand, reduced renal expression of CIC-5 in microalbuminuric SHR has been previously demonstrated by Tanaka and Nakaki (55). These authors found that the relative expression of CIC-5 in the renal cortex of SHR was 60% lower than in Wistar-Kyoto (WKY) rats at 11 wk of age and 70% lower compared with 14-wk-old animals. Our findings are consistent with this previous study, since we have also found that the expression of the CIC-5 channel progressively decreases in the renal cortex of SHR.

One intriguing finding of our study is the occurrence of the dissociation between protein and mRNA expression of selected components of the endocytic machinery in the renal proximal tubule of SHR. The progressive decrease in the protein expression of megalin, cubilin, and CIC-5 in the renal cortex of SHR is not dependent on transcription regulation. In fact, protein expression decreased despite increased mRNA expression of these genes. This dissociation might be due to posttranscriptional events such as modulation of translation by RNA binding proteins (61) and/or by increased degradation of these membrane proteins. However, we have no direct explanation as to why this dissociation occurs.

Numerous in vitro studies (18, 19, 21) as well as in vivo observations (22) have demonstrated that NHE3 is one of the plasma membrane transport proteins necessary for albumin endocytosis in the renal proximal tubule. The progressive decrease in the protein expression of megalin, cubilin, and CIC-5 in the renal cortex of SHR remains to be determined. We have previously demonstrated that NHE3 transport activity is inhibited in the renal proximal tubule of SHR after development of hypertension (12). Inhibition of NHE3-mediated NaHCO_3 reabsorption in 14-wk-old SHR compared with age-matched WKY rats is mainly due to redistribution of the transporter from the body to the base of the microvilli with no significant changes in NHE3 total protein abundance. Furthermore, changes in NHE3 transport activity were accompanied by changes in the phosphorylation level of the transporter at the PKA consensus site serine 552 (12, 23, 35). In this regard, receptor-mediated endocytosis has been shown to be downregulated by cAMP/PKA in proximal tubule cells via inhibition of NHE3 (21). Further work needs to be done to detect whether increases in the phosphorylation status of NHE3, especially at the PKA consensus sites, may lead to alkalinization of the endosomal compartment, and consequently to decreased uptake of albumin by proximal tubule cells.

There is a growing body of evidence suggesting that proximal tubular dysfunction may play an important role in the development of microalbuminuria in the early stages of diabetic nephropathy (51, 58). The tubular reabsorption of albumin is decreased in streptozotocin (STZ)-induced-diabetic-rats (58). This drop in albumin reabsorption occurs independently of changes in glomerular permeability (50, 51), and it is associated with a reduction of megalin expression in the renal proximal tubules (58). In a similar fashion, the results of our current study suggest that the causal events leading to microalbuminuria in hypertension are mainly of tubular origin. It is noteworthy to mention that microalbuminuria progressed to overt proteinuria in 48-wk-old SHR, when total urinary protein excretion was >500 mg/day. Moreover, analyses of urinary proteins by SDS-PAGE revealed that these 12-mo-old SHR excreted proteins with molecular weights both smaller and larger than albumin (data not shown). These observations suggest that dysfunction of the glomerular filtration barrier may occur at the macroalbuminuric stage. In this regard, previous studies have demonstrated that the integrity of the glomerular filtration barrier is normal in SHR during the first 9 mo of age. However, disturbances in glomerular protein sieving of a size-selective nature progressively develops in older hypertensive rats (3, 27).

In conclusion, these results demonstrate that the urinary excretion of albumin and transferrin, among other low-molecular-weight proteins, progressively increases in SHR from 6 to 21 wk of age and that this enhanced urinary protein excretion is associated with lower expression of key components of the apical endocytic apparatus in the renal proximal tubule. Moreover, it is tempting to speculate that dysfunction of the apical endocytic pathway in the renal proximal tubule may be a contributing mechanism underlying the development of microalbuminuria in hypertension.

REFERENCES

1. Agrawal V, Prasad N, Jain M, Pandey R. Reduced podocin expression in minimal change disease and focal segmental glomerulosclerosis is related to the level of proteinuria. Clin Exp Nephrol. [Epub ahead of print].


