Increased expression of ENaC subunits and increased apical targeting of AQP2 in the kidneys of spontaneously hypertensive rats

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THE EXPERIMENTAL MODEL OF spontaneously hypertensive rats (SHR) has been used to investigate the renal involvement in the pathogenesis of essential hypertension in humans. Altered renal functions have been suggested to play a role in the pathogenesis of essential hypertension. This has been documented by the development of high blood pressure in normotensive rats after renal cross-transplantation between normotensive and hypertensive strains where the genetic predisposition of hypertension is transferred with the donor kidney from the hypertensive strains. Moreover, this was demonstrated not only in adult rats with established hypertension (6) but also in young prehypertensive rats (32). These data therefore support the hypothesis that altered renal functions contribute significantly to the development and maintenance of primary hypertension. The renal defects could range from the altered glomerular hemodynamics to abnormal regulation of renal tubular sodium and water transport.

The sodium (co)transporters and aquaporins (AQPs) expressed in kidney tubules play a critical role in the tubular sodium and water reabsorption and regulation of extracellular fluid (ECF) volume. Recently, our group (30, 45) demonstrated that altered expression of renal sodium transporters and AQPs is significantly associated with deranged urinary concentration, urinary sodium excretion, and abnormal ECF volume regulation in a variety of sodium and water balance disorders. Thus we hypothesize that the dysregulation of renal AQPs and sodium transporters expression could be important in the development and/or maintenance of hypertension.

In the kidney nephron, including collecting duct, Na-K-ATPase is expressed along the entire length of the basolateral membrane of the renal tubule and actively pumps sodium from the cell into the interstitium to set up the electrochemical gradient to allow sodium to be reabsorbed. In the proximal tubule, the primary route for the apical sodium transport is via sodium/hydrogen exchanger type 3 (NHE3). In the thick ascending limb, apical sodium transport occurs via both NHE3 and bumetanide-sensitive Na-K-2Cl cotransporter (NKCC2 or BSC-1). In the distal convoluted tubule, sodium is primarily reabsorbed through the apical thiazide-sensitive Na-Cl cotransporter (NCC or TSC) (31). In the connecting tubule (CNT) through the collecting duct, sodium reabsorption across the apical plasma membrane occurs through epithelial sodium channel (ENaC). Particularly, the importance of ENaC in volume regulation has been demonstrated in recent studies that have identified mutations in ENaC as the basis of the pathogenesis of Liddle’s syndrome, a disorder characterized by volume expansion and hypertension (50) as well as type I
pseudohypoaldosteronism, a disorder characterized by volume depletion and hypotension (9). ENaC is regulated by the adrenal mineralocorticoid hormone vasopressin, as well as by insulin, which both markedly increase the apical permeability of the collecting duct to sodium (7, 17, 35). AQP2 is the apical water channel of collecting duct principal cells and is the chief target for regulation of collecting duct water permeability by vasopressin (45). Altered expression and apical targeting of AQP2 have been shown to play a significant role in water balance disorders (45).

In models of genetic (primary) hypertension, the renal involvement may be presented, at least in part, by inappropriate sodium and water retention (5). The excessive retention of sodium in SHR may result from either a reduced glomerular filtration rate (GFR) (15, 53) or an enhanced tubular reabsorption (40) or both. The activities of Na-K-ATPase and sodium/hydrogen exchanger in the proximal tubule was found to be significantly higher in young SHR compared with age-matched Wistar-Kyoto rats (WKY) (21, 40), and these changes may account in part for the increased ECF volume expansion in SHR. However, the roles of other tubule segments and the molecular basis for the inappropriate sodium and water retention remain largely undefined in SHR. It should also be emphasized that the difference found in different ages of SHR support the view that dynamic changes occur that likely represent changes of importance of the onset or maintenance of hypertension or represent compensatory changes.

We therefore hypothesize that dysregulation of renal ENaC subunits, sodium transporters, or AQP2 may be involved in the pathogenesis of hypertension in SHR. We performed semiquantitative immunoblotting and immunohistochemistry with the following specific purposes: 1) to examine whether there are changes in the abundance and/or apical membrane targeting of ENaC subunits in SHR, 2) to examine whether there are changes in the abundances of other important renal sodium transporters (e.g., Na-K-ATPase, NHE3, NKCC2, NCC), 3) to examine whether there are changes in the abundance and/or apical membrane targeting of AQP2, and 4) to examine whether these changes are associated with changes in the urinary sodium excretion, urinary concentration, and hypertension in SHR.

METHODS

Experimental animals. Male SHR and WKY rats (6 wk and 12 wk of age) were used for experiments. Five-week-old male SHR and WKY rats were purchased from Charles River Breeding Laboratories (Wilmington, MA). The animal protocols were approved by the boards of the Institute of Anatomy and Institute of Clinical Medicine, University of Aarhus, in accordance with the licenses for use of experimental animals issued by the Danish Ministry of Justice.

Experimental protocols. The rats were maintained in individual cages (one rat in each cage) in a room with an ambient temperature and a 12:12-h light-dark cycle, a temperature of 21 ± 2°C, and humidity of 55 ± 2%. Rats were maintained on a standard rodent diet (Altromin 1324, Lage, Germany). In the WKY group, rats were offered the amount of food corresponding to the mean intake of food that SHR consumed during the previous day. Thus the food intake was matched between the two groups. Both SHR and WKY rats were allowed to have free access to water intake at all times. During the last 3 days, the rats were subsequently maintained in the metabolic cages to allow urine collections for the measurements of protein, Na+, K+, creatinine, and osmolality. Water and food intake and body weight were monitored. The daily 24-h urine output and water intake were measured. The rats were killed for immunohistochemistry and immunohistochemical studies at 6 or 12 wk of age. Before being killed, the rats were anesthetized with isoflurane (Forane; Abbott Laboratories, Gentofte, Denmark). PE-50 catheters were introduced into the abdominal aorta for recording blood pressure (Sirecust 961 and Siredoc 220; Siemens, Munich, Germany). Blood was collected from the inferior vena cava and analyzed for sodium, potassium, creatinine, and osmolality. The right kidney was rapidly removed, dissected into three zones [cortex and outer stripe of outer medulla (cortex/OSOM), inner stripe of outer medulla (ISOM), and inner medulla], and processed for semiquantitative immunoblotting, as described below. The left kidney was fixed by retrograde perfusion as described below.

Clearance studies. Clearance studies were performed over the last 24 h. The plasma concentrations of sodium, potassium, and creatinine and the urinary concentration of creatinine were determined (Vitros 950, Johnson & Johnson). The concentrations of urinary sodium and potassium were determined by standard flame photometry (Eppendorf FC6341). The osmolalities of urine and plasma were determined by freezing point depression (Advanced Osmometer, model 3900, Advanced Instruments, Norwood, MA; and Osmomat 030-D, Gonotec, Berlin, Germany).

Plasma aldosterone concentrations were determined using a commercially available radioimmunoassay kit (Coat-A-Count; Diagnostic Products, Los Angeles, CA).

Semiquantitative immunoblotting. The dissected renal cortex/ISOM, ISOM, and inner medulla were homogenized (Ultra-Turrax T8 homogenizer; IKA Labortechnik, Staufen, Germany) in ice-cold isolation solution containing 0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, 8.5 mM leupeptin, and 1 mM phenylmethylsulfonyl fluoride, with pH 7.2. The homogenates were centrifuged at 4,000 g for 15 min at 4°C to remove whole cells, nuclei, and mitochondria, and the supernatant was pipetted off and kept on ice. The total protein concentration was measured (bicinchoninic acid protein assay reagent kit; Pierce, Rockford, IL). All samples were adjusted with isolation solution to reach the same final protein concentrations and solubilized at 65°C for 15 min in SDS-containing sample buffer and then stored at −20°C. To confirm equal loading of protein, an initial gel was stained with Coomassie blue. SDS-PAGE was performed on 9 or 12% polyacrylamide gels. The proteins were transferred by gel electrophoresis (Bio-Rad Mini Protein II) onto nitrocellulose membranes [Hybond enhanced chemiluminescence (ECL) RPN3032D; Amersham Pharmacia Biotech, Little Chalfont, UK]. The blots were subsequently blocked with 5% milk in PBS-Tween 20 (80 mM NaH2PO4, 20 mM Na2HPO4, 100 mM NaCl, and 0.1% Tween 20, pH 7.5) for 1 h and incubated overnight at 4°C with primary antibodies. The sites of antibody-antigen reaction were visualized with horseradish peroxidase-conjugated secondary antibodies (P447 or P448, diluted 1:3,000; Dako, Glostrup, Denmark) with an ECL system (ECL or ECL+plus) and exposure to photographic film (Hyperfilm ECL; Amersham Pharmacia Biotech). The band density was quantitated by scanning the films, and labeling density was corrected by densitometry of Coomassie-stained gels. Results are presented as the relative abundances between the groups. ECL films with bands within the linear range were scanned using an AGFA scanner (ARCUS II). The labeling density was corrected by densitometry of Coomassie-stained gels run in parallel to the blotted gels.

Immunohistochemistry. Kidneys were fixed by retrograde perfusion via the aorta with 3% paraformaldehyde, in 0.1 M cacodylate buffer, pH 7.4. Immunolabeling was performed on sections from paraffin-embodied preparation (2 μm thickness) using methods described previously in detail (30).

Primary antibodies. Rabbit polyclonal antibodies to the following renal sodium transporters were utilized (30): NHE3, NKCC2, NCC, and the ENaC subunits α-ENaC, β-ENaC, and γ-ENaC. In addition, we used previously characterized rabbit polyclonal antibodies to AQP2 (44). A mouse monoclonal antibody against the Na-K-ATPase...
α1-subunit was kindly provided by Dr. D. M. Fambrough (Johns Hopkins University Medical School).

Statistical analyses. Values are presented as means ± SE. Comparisons between age-matched WKY and SHR were made by unpaired t-test. Multiple comparisons among the groups (WKY and SHR in both 6- and 12-wk ages) were analyzed by one-way ANOVA followed by Tukey’s honestly significantly different multiple comparisons test. Multiple comparisons tests were only applied when a significant difference was determined in the ANOVA, P < 0.05. P values < 0.05 were considered significant.

RESULTS

Creatinine clearance and urinary sodium excretion rate were decreased in SHR. SHR at 6 and 12 wk of age exhibited lower body weight compared with corresponding age-matched WKY animals (Tables 1 and 2). Systolic blood pressure was significantly higher in both 6-wk SHR (Table 1) and 12-wk SHR (Table 2). Accordingly, mean arterial pressure was increased. The urine output was markedly decreased in both 6- and 12-wk SHR. Plasma creatinine level was not changed, whereas renal creatinine clearance was decreased in both 6- and 12-wk SHR. Plasma levels of sodium and potassium were unchanged in SHR. The 24-h urinary sodium excretion rate was decreased in both 6- and 12-wk SHR. However, fractional excretion of sodium (FENa) and fractional excretion of potassium were not changed. The urinary osmolality and urine-to-plasma osmolality ratio were significantly increased in both 6-wk and 12-wk SHR, indicating an increased urinary concentration (Tables 1 and 2). Plasma aldosterone level was not changed in both 6-wk (397.7 ± 68.1 pg/ml in SHR vs. 390.1 ± 80.0 pg/ml in WKY; not significant) and 12-wk SHR (850.6 ± 46.9 pg/ml in SHR vs. 779.8 ± 69.2 pg/ml in WKY; not significant) compared with age-matched WKY.

Increased and maintained protein expression of ENaC subunits in 6- and 12-wk SHR. Semiquantitative immunoblotting was carried out to investigate whether the abundance of ENaC subunits changes in SHR. In 6-wk SHR, the protein abundance of β-ENaC was markedly increased in the cortex/OSOM (482 ± 99 vs. 100 ± 32%; P < 0.05) and ISOM (540 ± 55 vs. 100 ± 23%; P < 0.05) compared with age-matched WKY, but was not altered in the inner medulla (Fig. 1). The abundance of γ-ENaC was also significantly increased in the cortex/OSOM (164 ± 6 vs. 100 ± 6%; P < 0.05) and ISOM (137 ± 4 vs. 100 ± 6%; P < 0.05) but was not altered in the inner medulla (Fig. 1). The α-ENaC abundance was increased in the ISOM (163 ± 12 vs. 100 ± 17%; P < 0.05) but was not changed in the cortex/OSOM and inner medulla (Fig. 1). In 12-wk SHR, the protein abundance of β-ENaC (161 ± 7 vs. 100 ± 7%; P < 0.05) and γ-ENaC (133 ± 4 vs. 100 ± 4%; P < 0.05) was increased in the cortex/OSOM but was not changed in the ISOM and inner medulla (Fig. 2). The α-ENaC abundance remained unaltered in the cortex/OSOM, ISOM, and inner medulla (Fig. 2). One-way ANOVA with multiple comparison test revealed that the increased abundance of β-ENaC was more prominent in the cortex/OSOM and ISOM in 6-wk SHR compared with 12-wk SHR (P < 0.01). Moreover, the increased abundance for γ-ENaC was more prominent in the ISOM, not in the cortex/OSOM, in 6-wk SHR compared with 12-wk SHR (P < 0.01).

Immunoperoxidase microscopy of β-ENaC and γ-ENaC subunits. In addition to regulation of protein abundance of the ENaC subunits, ENaC is also regulated by intracellular trafficking. To investigate whether the trafficking of ENaC subunits is altered in SHR, we carried out immunoperoxidase microscopy of β-ENaC and γ-ENaC subunits. In 6-wk WKY, β-ENaC subunits showed diffuse cytoplasmic labeling throughout the late distal convoluted tubule (DCT2; Fig. 3A), CNT (Fig. 3C), and collecting duct principal cells (Fig. 3, E and G), as previously observed (30). In 6-wk SHR, immunoperoxidase staining for the β-ENaC subunit was similar to the corresponding WKY results and the labeling was mainly cytoplasmic (Fig. 3, B, D, and F), indicating that subcellular localization was not changed. In contrast β-ENaC labeling intensity was markedly increased in the principal cells of DCT2 (Fig. 3B), CNT (Fig. 3D), cortical collecting duct (CCD; Fig. 3F), and outer medullary collecting duct (not shown), consistent with the immunoblotting analysis (Fig. 1).

Immunohistochemical pattern of γ-ENaC in 6-wk SHR was similar to that of β-ENaC. Compared with 6-wk WKY (Fig. 4,
A, C, E, and G), immunolabeling intensities for the γ-ENaC in 6-wk SHR in the DCT2 (Fig. 4B), CNT (Fig. 4D), CCD (Fig. 4F), and outer medullary collecting duct (not shown) were increased. These results are consistent with the increased γ-ENaC protein abundance observed by immunoblotting (Fig. 1). In contrast, subcellular localization was mainly cytoplasmic in 6-wk SHR (Fig. 4, B, D, and F) and similar to the age-matched WKY (Fig. 4, A, C, and E).

Immunohistochemical analysis in 12-wk SHR revealed similar changes in the β-ENaC and γ-ENaC, such as shown in 6-wk SHR. Immunoperoxidase microscopy revealed increased immunolabeling intensity of β-ENaC in 12-wk SHR in DCT2 (not shown), CNT (Fig. 5B), and CCD (Fig. 5D) compared with age-matched WKY (Fig. 5, A and C), whereas the subcellular localization was similar to that shown in age-matched WKY (Fig. 5, A–D). The immunolabeling pattern was qualitatively the same for γ-ENaC. The immunolabeling intensity of γ-ENaC was significantly increased in DCT2 (not shown), CNT (Fig. 5F), and CCD (not shown) compared with age-matched WKY (Fig. 5E), whereas the subcellular localization was not changed (Fig. 5, E and F).

Fig. 1. Semiquantitative immunoblots of kidney proteins prepared from cortex/outer stripe of outer medulla (OSOM), inner stripe of outer medulla (ISOM), and inner medulla from 6-wk-old spontaneously hypertensive rats (SHR) and age-matched Wistar-Kyoto control rats (WKY). Protein abundance of β-ENaC and γ-ENaC (ENaC is defined as epithelial sodium channel) was markedly increased in the cortex/OSOM and ISOM but unchanged in inner medulla. Protein abundance of α-ENaC was increased in ISOM but was not changed in the cortex/OSOM and inner medulla. *P < 0.05.

Fig. 2. Semiquantitative immunoblots of kidney proteins prepared from cortex/OSOM, ISOM, and inner medulla from 12-wk-old SHR and age-matched WKY. Protein abundance of β-ENaC and γ-ENaC was markedly increased in the cortex/OSOM but unchanged in ISOM and inner medulla. α-ENaC abundance remained unaltered in the cortex/OSOM, ISOM, and inner medulla. *P < 0.05.

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Increased Na-K-ATPase abundance in the cortex/OSOM in 6-wk SHR. Semiquantitative immunoblotting revealed an increase in Na-K-ATPase abundance in the cortex/OSOM (173 ± 14 vs. 100 ± 15%; P < 0.05), although it remained unchanged in the ISOM and inner medulla in 6-wk SHR (Fig. 6). The protein abundances of NHE3, NKCC2, and NCC were not changed in the kidneys from 6-wk SHR and WKY (Fig. 6). Consistent with this, the protein abundances of the renal sodium transporters (Na-K-ATPase, NHE3, NKCC2, and NHE3) were not changed in the renal cortex/OSOM, ISOM, and inner medulla in 12-wk SHR compared with age-matched WKY (not shown).

Increased immunolabeling of Na-K-ATPase in the proximal tubule and CCD from 6-wk SHR. We carried out the immunoperoxidase microscopy for the α1-isoform of Na-K-ATPase to investigate whether there were changes of the immunolabeling of the Na-K-ATPase in the basolateral membranes of the renal tubular segments. Immunoperoxidase labeling of the α1-isoform of Na-K-ATPase revealed that immunolabelings in proximal tubule, CCD, and outer medullary collecting duct were markedly increased in 6-wk SHR (Fig. 7, B and D) compared with that shown in age-matched WKY (Fig. 7, A and C). In contrast, the immunolabeling of Na-K-ATPase in the thick ascending limb (Fig. 7) and inner medullary collecting duct (not shown) was unchanged in 6-wk SHR compared with age-matched WKY. In 12-wk SHR and age-matched WKY, the immunolabeling of Na-K-ATPase in the proximal tubule and collecting duct exhibited similar labeling intensity (not shown).

Increased protein expression and apical targeting of AQP2 in SHR. Semiquantitative immunoblotting revealed an increase in AQP2 abundance in the ISOM (136 ± 6 vs. 100 ± 4%, P < 0.05; Fig. 8) in 6-wk SHR compared with age-matched WKY but was not changed in the cortex/OSOM and inner medulla (Fig. 8). In contrast, protein abundances of AQP2 in 12-wk SHR were not changed in the cortex/OSOM, ISOM, and inner medulla (not shown). To investigate whether the intracellular trafficking of AQP2 to the apical plasma membrane was enhanced in SHR, we carried out immunoperoxidase microscopic analyses. Immunolabeling of AQP2 was seen in the collecting duct principal cells. There was a prominent difference in the subcellular localization of AQP2 in kidneys from SHR and WKY. AQP2 labeling in the inner medullary collecting ducts of the kidneys from 6-wk WKY was mainly associated with cytoplasm, and less labeling was observed at the apical plasma membrane (Fig. 9A). In contrast, AQP2 labeling was seen predominantly localized to the apical plasma membrane domains, and only marginal cytoplasmic labeling was seen in 6-wk SHR (Fig. 9B). Immunohistochemical analysis also revealed an increased apical labeling of AQP2 in inner medulla of kidneys from 12-wk SHR (Fig. 9D) compared with the 12-wk WKY (Fig. 9C).

DISCUSSION

In this study, we examined the changes in protein expression levels of renal sodium transporters, ENaC subunits, and AQP2.
in SHR at two different stages (early and late hypertensive). The increases in abundance of ENaC subunits and α1-subunit of Na-K-ATPase would be predicted to result in enhanced tubular sodium reabsorption in early stage (6 wk) in SHR. In addition, the increased apical membrane targeting of AQP2 in the collecting duct may play a role in the increased water reabsorption. It is noteworthy that the increased abundance of ENaC subunits was more prominent in early stage of 6-wk SHR than in late hypertensive stage of 12-wk SHR, and the Na-K-ATPase immunolabelings in both the proximal tubule and the collecting duct were significantly increased in 6-wk SHR but not in 12-wk SHR. The prominent changes of increased ENaC abundance in association with the increased Na-K-ATPase expression in the renal collecting duct may at least in part play a significant role for the increased sodium reabsorption in early stages of SHR and hence induce the increased circulating volume and development of hypertension. The increased expression of Na-K-ATPase in proximal tubule may potentially also play a role. These findings therefore raise the possibility that the sodium-retaining effect in the collecting duct and proximal tubule is prominent in early stage of SHR and that the effect is gradually attenuated in late hypertensive stage of SHR (15). As a result, these alterations in the protein abundance of sodium transporters and/or AQP2 water channel might play roles in the development and/or maintenance of elevated blood pressure in SHR.

Decreased GFR and urinary sodium excretion in SHR. The present study revealed that the systolic blood pressure and mean arterial blood pressure were higher in 6-wk-old SHR than in age-matched WKY, even though they did not develop significant hypertension, although significant hypertension was observed in 12-wk SHR. The pattern of blood pressure rise in the SHR during the first 10 wk of age is disputed or variable. Some studies did not observe an increased blood pressure until 6 wk of age; others (22) found high blood pressure even in the newborn SHR. The gradual increase in blood pressure from 6 wk of age seen in the present study is supported by several studies (11, 33).

An important mechanism by which the kidney contributes to the regulation of long-term blood pressure is altered renal sodium handling to maintain the sodium-retaining properties (12). The present study indicated that, already at the age of 6 wk, renal function was substantially changed and that these changes were mostly a consequence of a reduced GFR, causing a lesser amount of sodium to be filtered and excreted. This is consistent with the idea that kidneys in SHR excrete less sodium than those of WKY when blood pressure increases, i.e., especially between weeks 6 and 12 of age (5). The reduced GFR in young SHR is in agreement with previous results (15); thus the decreased GFR impaired the natriuretic capacity in SHR. On the other hand, no significant difference in FENa was seen in both ages of SHR. However, when we consider the high perfusion pressure in SHR, the unchanged FENa may suggest the relatively increased sodium reabsorption in the renal tubules.
It is well known that pressure-natriuresis is the mechanism by which changes in arterial pressure are followed by changes in renal sodium excretion. In such a way, chronic hypertension cannot be sustained without the active participation of the kidney to increase reabsorption of sodium and water. Otherwise, elevated renal perfusion pressure leads to salt and water diuresis and returns blood pressure to normal levels (23). Consistent with this, Arendshorst and Beierwaltes (2) reported that SHR exhibited a diminished ability to excrete sodium and water compared with the age-matched WKY when renal perfusion pressure was lowered to normotensive levels by aortic constriction. Similar results have been reported in the stroke-prone strain of SHR (43) and in hypertensive patients whose pressure was lowered with an infusion of nitroprusside (46). The reason for the decreased ability of the kidneys to excrete sodium and water in hypertensive rats and patients when kidneys are perfused at normotensive pressure has not been determined. We hypothesize that the decreased ability of the kidneys to excrete sodium and water at equivalent perfusion pressure might be related to increased tubular reabsorption in SHR.

In a physiological setting, one would predict that no changes would occur in sodium balance with changes in GFR since the fractional reabsorption of sodium will change in parallel. A recent paper from Schnermann and associates (24) illustrated this nicely in AQP1 knock-out mice (with severe reduction in GFR and polyuria) and in AQP1 and adenosine 1 receptor double knock-out mice (with normal GFR, increased distal sodium and fluid delivery, and polyuria), exhibiting no change in urinary sodium excretion between them. Thus we hypothesize that the overall defects in sodium balance is likely to involve changes in sodium channel and/or sodium transporter expression or activity. This may then potentially be enforced by reduction in GFR. Increased abundance of ENaC subunits in SHR. In the present study, the abundance of ENaC subunits was significantly increased in both ages of SHR; however, the observed increase in late hypertensive stage (12 wk) was at a lesser degree than that in the early stage (6 wk). The protein abundance of β-ENaC and γ-ENaC was consistently increased in 12-wk-old SHR. γ-ENaC immunolabeling was also significantly increased in the CNT (E and F).

**Fig. 5.** Immunoperoxidase microscopy of β-ENaC and γ-ENaC in CNT and CCD in 12-wk-old SHR and WKY. Immunoperoxidase staining for the β-ENaC and γ-ENaC showed diffuse punctuate labeling throughout the CNT and CCD principal cells in both 12-wk-old SHR and WKY. Immunolabeling of β-ENaC revealed increased labeling in the principal cells of CNT (A and B) and CCD (C and D) in 12-wk-old SHR. γ-ENaC immunolabeling was also significantly increased in the CNT (E and F).
that there may have been transient changes in enhanced ENaC trafficking during the initial stage of SHR, and only increased protein abundance may prevail.

The ENaC channel activity is regulated by a number of mechanisms, including aldosterone-stimulated intracellular trafficking of the ENaC α-, β-, and γ-subunits from the cytoplasm to the apical plasma membrane (35). Because the synthesis of the α-ENaC subunit has been suggested to be a rate-limiting factor of the multimeric ENaC complex, sodium transport could be expected to be proportional to the abundance of the α-ENaC protein levels. The role of the β- and γ-subunit is uncertain. Neither the β- nor the γ-subunit, when expressed alone or together, has been shown to produce any measurable Na⁺ current, whereas coexpression with the α-subunit greatly enhanced the amplitudes of the Na⁺ current (8, 50). These results were interpreted to mean that the β- and γ-subunits probably have a structural and/or regulatory role in the stabilization and function of the channel. In the studies of Djelidi et al. (16), in vitro incubation of the cells with vasopressin resulted in a rapid and sustained (up to 10 h) increase in amiloride-sensitive short-circuit current and sodium transport. The increased ENaC activity and sodium transport were associated with increased expression of β-ENaC and γ-ENaC subunits in the rat CCD cell line in the absence of changes in α-ENaC. Moreover, gene knock-out mice for either β-ENaC or γ-ENaC subunit revealed a significant phenotype: very high urinary sodium excretion, low urinary potassium excretion, severe hyperkalemia, and death before adulthood (3, 38). Thus the observed significant increase of β-ENaC and γ-ENaC subunits in association with maintained or marginally increased α-ENaC in SHR may contribute significantly to the increased renal tubular sodium reabsorption and the pathogenesis of development and/or maintenance of hypertension.

It has been demonstrated that ENaC activity and expression are regulated by several hormones, such as vasopressin (17), aldosterone (35), and insulin (7). Long-term increases in circulating aldosterone concentrations result in increases in the abundances of the α-subunit protein with decreased abundance of β-subunit protein and appearance of a 70-kDa γ-ENaC band with a concomitant decrease in the main 85-kDa band (37). In contrast, long-term exposure to high circulating vasopressin levels stimulates a marked increase in the abundances of the β- and γ-subunit proteins, with little or no effect on the abundance of the α-subunit (17). Thus the change of the ENaC subunits in SHR in the present study is likely to be the effect of vasopressin rather than aldosterone (13, 27, 42, 53).

Most studies indicate low or normal plasma aldosterone levels (14, 20, 28), and the present study also revealed that plasma aldosterone levels were not changed in early (6 wk) and late hypertensive (12 wk) SHR rats compared with age-matched WKY rats. The absence of a significant increase of plasma renin concentrations and the failure to lower blood pressure by saralasin infusion suggest that the renin-angiotensin system plays a minor or no role in maintaining the hypertension in SHR (39).

**Unchanged expression of NKCC2 and NCC in SHR.** An elevation in chloride reabsorption in the thick ascending limb of the loop of Henle by overexpression of NKCC2 has been suggested as a pathogenic mechanism of hypertension in Dahl salt-sensitive rats and prenatal programmed hypertension (26, 36). Thus the role of NKCC2 in the development of hypertension in SHR has been suggested. However, no conclusive data are available in SHR so far. Isolated and perfused SHR kidneys showed a lower sensitivity to furosemide than their control WKY (47). Taken together with our results of unchanged NKCC2 abundance and increased abundance of ENaC subunits, the previously demonstrated decreased responsiveness to furosemide suggest that increased sodium reabsorption in kidneys of SHR occurs primarily in the collecting duct but not in the thick ascending limb.

Some studies have pointed to the NCC as a potential candidate in the development of hypertension. Beaumont et al. (4), found that the thiazide receptor in the renal cortex, assessed by
[3H]metolazone-binding analysis, was normal at birth and 4 wk of age in SHR, but the binding increased at 14, 35, and 49 wk of age, as well as the development of hypertension, suggesting the enhanced activity of NCC in adult SHR. However, the mRNA level of NCC was not changed at 4, 10, and 16 wk of age in SHR (41). In agreement with this, our results also revealed that the protein expression of NCC was not altered and plasma aldosterone levels were not changed in SHR at 6 and 12 wk of age. Also this was consistent with previous studies demonstrating normal plasma aldosterone levels (20, 28) and normal aldosterone binding in the whole kidney of SHR (19). Thus NCC changes in the gene transcription rate or protein translation rate are not implicated in the development of hypertension.

Increased immunolabeling of Na-K-ATPase in the proximal tubule and collecting duct from 6-wk-old SHR. In the proximal tubule, sodium is absorbed via apical transporters, including the NHE3. The electrochemical gradient driving sodium entry is provided by basolateral Na-K-ATPase, which extrudes sodium into the extracellular space. Isolated and perfused tubule preparations and micropuncture experiments have shown that proximal tubule of early SHR reabsorbed more sodium than WKY (1, 54). Dramatic increases in sodium/hydrogen exchange activity have also been reported in proximal tubule cells of early SHR, but only a modest increase (33) or no changes (34) in protein abundance were observed. Consistent with this, we demonstrated that NHE3 expression was not changed in both ages of SHR compared with the corresponding age-matched WKY. In contrast to the unchanged abundance of NHE3, protein abundance of α1-subunit of Na-K-ATPase was increased in the renal cortex/OSOM in 6-wk SHR but was not changed in 12-wk SHR. In addition, immunolabeling for the Na-K-ATPase in the proximal tubule was increased in 6-wk-old SHR compared with age-matched WKY. These findings are consistent with the previous results that indicated that proximal tubules of early SHR but not late hypertensive SHR have increased Na-K-ATPase activity (21). In the CNT and collecting duct, the apical sodium entry pathway is accounted for by ENaC and intracellular sodium is then extruded by basolateral Na-K-ATPase, which provides the driving force for sodium reabsorption. In the present study, Na-K-ATPase immunolabeling in the collecting duct was also significantly increased in 6-wk-old SHR, associated with the increased abundance of ENaC subunits. Thus enhanced sodium reabsorption in the collecting duct may be activated by coordinated function of ENaC and Na-K-ATPase in 6-wk-old SHR rats.

Fig. 7. Representative immunoperoxidase finding for Na-K-ATPase α1-subunit in the cortex (A and B) and ISOM (C and D) in 6-wk-old SHR and WKY. Immunolabeling for Na-K-ATPase in the PT, CCD, and outer medullary collecting duct (OMCD) was increased in 6-wk SHR (B and D) compared with that shown in WKY (A and C). In thick ascending limb of Henle (TAL), staining for Na-K-ATPase antibody was comparable between 2 groups. Magnification: ×250 (A and B), ×630 (C and D).

Increased apical targeting of AQP2 in the inner medullary collecting duct. During the development of hypertension in the SHR, it is likely that both mechanisms involved in the cause of the hypertension as well as mechanisms of compensatory role could be activated. From the literature, we have chosen two
time points to be important: an early stage and a later stage with profound hypertension. Thus it is possible that the increased AQP2 expression at 6 wk but not at 12 wk may play a role in the development of hypertension and that this may be related to increased plasma vasopressin.

The effects of chronic blockade of the vasopressin receptors during hypertension of SHR appear controversial and the role of the V1 receptor in the pathogenesis of hypertension is unclear (51, 52, 55). It has been demonstrated that increased plasma vasopressin levels and enhanced binding capacity in the kidney tubules induce the decreased urine volume and increased urine osmolality (27, 53) and that they play roles in the pathogenesis of hypertension by expanding effective circulating blood volume via the V2 receptor. The radiolabeled receptor assay of the medulla membrane fraction revealed that the binding capacity for the V2 receptor in 8- and 12-wk-old SHR was significantly increased but was not changed in 3-wk-old and 7-wk-old SHR (27, 53). These findings suggest that SHR have different developmental changes in kidney vasopressin receptors and that renal V2 receptors play roles in maintaining fluid homeostasis in SHR. In the present study, both ages of SHR revealed decreased urine volume and increased urine osmolality compared with age-matched WKY. Consistent with this, immunohistochemical analysis revealed a markedly increased apical targeting of AQP2 in SHR at both ages, although the degree of increase was more prominent in 12-wk-old SHR than in 6-wk-old SHR. The prominent apical targeting of AQP2 in 12-wk-old SHR can be supported by the increased responsiveness to vasopressin in hypertensive stages (27).

The protein abundance of AQP2 was significantly increased in ISOM in 6-wk-old SHR, suggesting that upregulation of the vasopressin-regulated water channel AQP2 may contribute to the increased urinary concentration and water reabsorption seen in 6-wk-old SHR. However, the functional significance of the selective increase in AQP2 expression in ISOM remains unknown. Studies in isolated collecting duct cells suggest that the presence of mineralocorticoid is needed for a normal action of vasopressin on transepithelial osmotic water permeability (10, 48, 49). Furthermore, recently Hasler et al. (25) demonstrated that chronic aldosterone stimulation in the presence of vasopressin significantly increased AQP2 expression in mouse collecting duct cells, whereas collecting duct cells treated with aldosterone alone did not exhibit any change in AQP2 expression. In addition, the mineralocorticoid receptor antagonist canrenoate abolished upregulation of AQP2 protein (29). These findings suggest that aldosterone coordinated with vasopressin can modulate the water reabsorption in collecting duct principal cells. Because it has been shown that aldosterone binding was higher in the medullary collecting duct but not in the CCD in SHR (18), it is plausible that the increased AQP2 expression in the medullary collecting duct in 6-wk-old SHR can be explained by the different local sensitivities to vasopressin and aldosterone.

In summary, the results demonstrate that the protein abundance of ENaC subunits was increased in DCT2, CNT, and collecting duct segments at both ages of SHR studied, of which the degree was more prominent in 6-wk-old SHR compared with 12-wk-old SHR. The protein abundance of Na-K-ATPase was increased in the renal cortex in 6-wk SHR but not in 12-wk SHR. Immunoperoxidase microscopy revealed that Na-K-ATPase labeling was increased in both the proximal tubule and the cortical and outer medullary collecting duct in 6-wk-old SHR. Moreover, there was an increased apical targeting of AQP2 in the inner medullary collecting duct, associated with increased or sustained abundances of AQP2 expression in SHR. In contrast, expression of NHE3, NKCC2, and NCC was not altered in SHR. These findings suggest the increased protein abundance of ENaC subunits and Na-K-ATPase as well as the increased apical targeting of AQP2 may contribute to the retention of sodium and water and the development of hypertension in SHR.

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REFERENCES


