Altered Regulation of Epithelial Sodium Channel in the Kidney in Rats with Sodium Retention

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Localization of sodium (co)transporters and epithelial sodium channels (ENaC) in the kidney

In the kidney nephron, 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)[1]. In the collecting duct, electrogenic entry of sodium from the lumen into the cells is mediated by ENaC located in the apical plasma membrane[1]. This represents the rate limiting step for sodium absorption and is characterized by inhibition with submicromolar concentrations of the diuretic amiloride[2]. Gain of function mutations causes Liddle's syndrome, an inherited form of salt retention causing hypertension[3, 4]. Conversely, loss of function mutations causes pseudohypoaldosteronism type 1 (PHA), characterized by salt wasting and hypotension[5]. Thus, ENaC has a critical role in the maintenance of sodium homeostasis.

In rat kidney, the three homologous subunits (α, β, and γ) that constitute the functional ENaC protein are detected in the late distal convoluted tubule (DCT2), connecting tubule, cortical (CCD) and outer medullary collecting duct (OMCD), and to a lesser extent, the inner medullary collecting duct (IMCD). It has been shown that αENaC is mainly present at the apical domains of the principal cells, whereas β-and γ ENaC are mainly associated with intracellular vesicles dispersed in the entire cytoplasm[6-8]. The physiological significance of the heterogeneity in the subcellular localization of the three subunits has not been established. The α-subunit is functional when expressed alone in *Xenopus* oocyte, but the channel activity is highly increased by association with β- and γ-subunits[9]. The generation of gene knock-outs of the individual subunits in mice has demonstrated that altered expression of any of the three subunits has significant effects on the multimeric ENaC protein sodium transport capacity[10]. Regulation of sodium reaborption by ENaC mediated by hormones such as aldosterone and vasopressin is associated with characteristic alterations in the expression of the individual ENaC subunits[11, 12]. Chronic aldosterone infusion in rats increases the protein abundance of αENaC. Moreover, aldosterone causes a mobility shift of γENaC from an 85 kDa band to 70 kDa band[12]. Chronic vasopressin infusion results in significantly increased abundances of all three ENaC subunits[11]. The apical plasma membrane expression of ENaC can also be altered by changes in the trafficking of the channel subunits

to the apical plasma membrane. Under sodium replete conditions, when aldosterone levels are low ENaC immunostaining of cortical collecting duct revealed diffuse labeling throughout the principal cells, consistent with localization in a vesicular pool[12]. In contrast, sodium restriction or aldosterone infusion caused a dramatic redistribution of ENaC to the apical membrane[12, 13]. These results suggest that not only protein abundance but also translocation of ENaC to the apical plasma membrane are under the tight control.

Pathophysiology of ENaC subunits in sodium retentive disease

The role of changes in the expression and/or subcellular localization has been investigated in sodium retentive diseases, including nephrotic syndrome, liver cirrhosis, heart failure and hypertension in which there is an inappropriate sodium retention.

1. Nephrotic syndrome

Nephrotic syndrome is a common manifestation of renal disease, and is associated with avid sodium retention leading to the development of edema and ascites. However, the mechanism for the sodium retention is still incompletely understood and the molecular basis remains undefined. Puromycin aminonucleoside (PAN)-induced nephrotic syndrome is one of the most extensively studied models of glomerulonephritis in rats. This experimental model mimics the minimal change disease glomerulopathy found in human pathology. The characteristic lesion in both the experimental model and human disease consists of vacuolation and flattened foot processes of podocytes. In addition to glomerular lesions, functional alterations of tubular transport have been demonstrated in PAN-treated animals. *In vivo* micropuncture studies in the unilateral model of PAN-induced nephrotic syndrome have shown that sodium reabsorption is specifically increased in the collecting duct and not in the proximal tubule and distal nephron[14]. Micropuncture of the accessible distal convoluted tubule revealed that the tubular sodium load was similar in PAN-treated kidney and control kidney of the same rat, and the final urine sodium excretion was threefold lower in urine collected from the PAN-treated kidney compared to that in the untreated control kidney in the same animal[14]. Thus, this study directly point to a role of increased sodium reabsorption in the collecting duct and, hence, it may be hypothesized that dysregulation of the key sodium channels and transporters in the collecting duct may be responsible for this. A few studies have subsequently demonstrated that sodium retention in PAN nephrotic rats is correlated with increased Na,K-ATPase activity and expression in the cortical collecting duct[15, 16].

We demonstrated that PAN-induced nephrotic syndrome is associated with ① sodium retention, decreased urinary excretion of sodium, a marked ascites, and increased plasma aldosterone level, ② upregulation of protein abundance of specific ENaC subunits in cortex, outer medulla and inner medulla, and ③ increased apical targeting of ENaC subunits in DCT2, CNT and collecting duct segments observed as increased immunolabeling in apical plasma membrane domains. In contrast, the protein levels of other major sodium transporters expressed in nephron segments at a site proximal to the connecting tubule (i.e., NHE3, NKCC2, Na,K-ATPase and NCC) were significantly reduced in these segments[7]. Indeed, there was no downregulation of the Na,K-ATPase expression in the collecting duct. Taken together, these observations therefore strongly support the view that the renal sodium retention associated with PAN-induced nephrotic syndrome is caused by increased sodium reabsorption in connecting tubule and collecting duct[17]. Thus, this is likely to represent a key molecular basis for the sodium

retention associated with PAN-induced nephrotic syndrome combined with the previously demonstrated increase in collecting duct Na,K-ATPase activity and protein abundance[15, 16]. Moreover, our results extend this study by demonstrating the segmental specific upregulation of all three ENaC subunits, and most importantly provide evidence for a significantly increased apical targeting by immunoelectron microscopy. The latter is essential since it is important to document that the increased expression is at the site of function i.e. in the plasma membrane. Finally, we also confirm that there is a uniform downregulation of all investigated sodium transporters in the TAL and proximal tubule. This together with observed increased expression and increased targeting of ENaC subunits in combination with the previous functional micropuncture studies strongly support the view that the sodium retention in the nephrotic syndrome may occur in the DCT2-CNT-collecting duct[7].

Nephrotic syndrome may develop as a result of primary diseases such as minimal change disease or immune glomerulonephritis. Membranous glomerulonephritis (MGN) remains the most common cause of primary nephrotic syndrome in adult. A further reason for its importance is that approximately 25 to 50% of patients progress to end-stage kidney disease over 10 years. Thus, MGN has a different and more progressive clinical course as compared to the non progressive benign character of minimal change nephrotic syndrome[18]. Mercury chloride has been known to induce a systemic autoimmune disease including membranous nephropathy with IgG deposits. This nephropathy is responsible for the development of high-range proteinuria and a full-blown nephrotic syndrome associated with generalized edema and ascites[19]. To elucidate whether the changes in expression and plasma membrane targeting of ENaC subunits in the PAN-induced minimal change nephrotic syndrome (caused by podocyte injury, not immune complex mediated) is unique to the PAN model or may be a more general characteristic of nephrotic syndrome including immune complex mediated glomerulonephritis, and to investigate the physiologic role of changes in subcellular distribution of ENaC subunits in the abnormal sodium retention and ascites formation during disease states, we examined the changes of ENaC abundance and/or trafficking and type II 11β hydroxysteroid dehydrogenase (11βHSD2) in $HgCl_2$ induced nephrotic syndrome in Brown Norway rats. The results demonstrated that $HgCl_2$-induced immune complex glomerulonephritis was associated with ① sodium retention, decreased urinary sodium excretion, development of ascites, and increased plasma aldosterone level; ② increased apical targeting of ENaC subunits in DCT2, CNT and collecting duct segments; and ③ decreased protein abundance of 11βHSD2[20]. These observations therefore strongly support the view that the renal sodium retention associated with $HgCl_2$ induced nephrotic syndrome is caused by increased sodium reabsorption in the distal nephron including the connecting tubule and collecting duct. These results are consistent with the results of our previous studies of nephrotic syndrome induced by puromycin aminonucleoside (PAN)[7]. Thus, a straightforward interpretation of these observations would be that increased apical targeting of ENaC subunits plays a role in the development of sodium retention in $HgCl_2$ nephrotic syndrome as well, and it could be argued that this mechanism in fact could be a general characteristic of the nephrotic syndrome including both minimal change disease and immune complex mediated glomerulonephritis.

Apical targeting of ENaC subunits and protein abundance of αENaC can be regulated by aldosterone[12, 13]. Indeed, plasma aldosterone levels were significantly increased in $HgCl_2$ nephropathy, and thus, it can be speculated that aldosterone stimulate the sodium reabsorption in $HgCl_2$

nephropathy. However, substantial evidence argue against a major role of aldosterone in nephrotic syndrome : ① ascites and edema occur in rats with nephrosis attributable to rabbit anti-rat kidney serum injection, without elevated aldosterone levels[21]. ② inhibition of angiotensin converting enzyme by captopril failed to induce natriuresis in rats with PAN nephrosis despite decreased aldosterone levels[22]. and ③ sodium retention was observed only in the affected proteinuria kidney in rats unilateral models of PAN nephrosis[14]. These observations suggest that aldosterone may not alone be involved in the sodium retention and presumably in the ENaC regulation in the nephrotic syndrome.

It has been suggested that the activity of 11βHSD2 can regulate the sodium reabsorption in the aldosterone responsive renal tubules by glucocorticoid induced activation of mineralocorticoid receptor (MR)[23]. We also demonstrate that there is a downregulation of 11βHSD2 protein expression in the kidney in $HgCl_2$ nephrotic syndrome as well as in PAN nephrosis. This finding is in line with recent studies in nephrotic patients [24] and in experimental nephrotic syndrome induced by PAN or adriamycin[25]. The investigators observed a rise in the plasma ratio of corticosterone/11-dehydrocorticosterone as well as in the urinary ratio of (THB+5α-THB)/THA in nephritic rats[25], and interpreted this change as a result of a decreased activity of 11β-hydroxysteroid dehydrogenase. We here propose that this may occur via a downregulated protein expression of 11βHSD2 in nephrotic syndrome. These findings suggest that downregulation of 11βHSD2 provides access of glucocorticoids to the MR, resulting in increased activity of MR and collecting duct sodium retention in rats with nephrotic syndrome.

The results demonstrate an increased apical targeting of ENaC subunits combined with diminished abundance of 11βHSD2 in the DCT2, connecting tubule and collecting duct[20]. This is likely to play a role for sodium retention associated with nephrotic syndrome in rats. Although it cannot be ruled out that the changes of 11βHSD2 are secondary to hyperkalemia and/or potentially associated acidosis, the results raises the possibility that downregulation of 11βHSD2 may in part play a role in increased activity of MR and collecting duct sodium retention in rats with nephrotic syndrome.

2. Liver cirrhosis

Renal sodium and water retention has been shown to be responsible for the development of ascites not only in patients with liver cirrhosis but also in experimental cirrhotic rats. Atrial natriuretic peptide resistance, arginine vasopressin (AVP), renin-angiotensin-aldosterone, and sympathetic nerve overactivity have been shown to modulate renal tubular functions and have been considered to be mainly involved in sodium and water retention in liver cirrhosis. In kidneys of cirrhotic rat, increased reabsorption of sodium and water in the distal nephron and collecting duct has been suggested to be one of the most important renal tubular dysfuctions involved in the pathogenesis of ascites. However, the underlying molecular and cellular mechanisms for the sodium retention are still incompletely understood.

In particular, the role of aldosterone in sodium retention and ascites formation in liver cirrhosis is still unclear. Previous studies demonstrated that plasma aldosterone levels were usually elevated in liver cirrhosis with ascites[26] and that spironolactone, a mineralocorticoid receptor antagonist, increased sodium excretion in these patients[27]. This suggests that hyperaldosteronism is of major importance in the pathogenesis of sodium retention. ENaC is the major sodium transport pathway in the collecting duct and both protein abundance and apical plasma membrane targeting of the ENaC-subunits are regulated by hormonal

stimulation e.g., aldosterone[12] and vasopressin[13]. We therefore speculate that altered expression and/or apical membrane targeting of ENaC subunits (α-, β-, and γ-subunits) may account for the increased sodium reabsorption in the collecting duct and sodium retention in liver cirrhosis.

1) CCl_4 induced liver cirrhosis

The renal responses for sodium retention displayed wide variations among the rats with CCl_4-induced liver cirrhosis. Some rats showed markedly decreased urinary sodium excretion (sodium retaining stage, group A) whereas the others exhibited unchanged urinary sodium excretion (maintenance stage, group B) compared with controls, even though all CCl_4-treated rats had significant amount of ascites. The results demonstrated that CCl_4-induced sodium retaining stage (group A) of liver cirrhosis was associated with ① decreased urinary sodium excretion, and increased or maintained plasma aldosterone levels; ② increased apical targeting of ENaC subunits in DCT2, CNT and collecting duct segments; and ③ decreased protein abundance of 11βHSD2. In contrast, maintenance stage (group B) of liver cirrhosis was associated with no changes in the urinary sodium excretion, trafficking and abundance of ENaC subunits and the abundance of 11βHSD2[28]. The most important finding is the striking increase in targeting of all ENaC subunits to the apical plasma membrane domain in DCT2, CNT and collecting duct in the sodium retaining stage (group A), but not in the maintenance stage (group B) of liver cirrhosis. Since an increased targeting of all ENaC subunits to the apical plasma membrane is associated with increased sodium reabsorption, the present finding of an increased ENaC targeting in the sodium-retaining stage of liver cirrhosis could contribute significantly to the increased renal tubular sodium reabsorption. These observations therefore strongly support the view that the renal sodium retention in the decompensated liver cirrhosis is mainly caused by an increased sodium reabsorption in distal nephron including the collecting duct[29].

We demonstrated that the abundance of β ENaC was decreased or unchanged, and the abundance of the 70-kDa form of γENaC was increased while the 85-kDa band was markedly decreased in the sodium-retaining stage (group A) of cirrhotic rats. Previous studies demonstrated that aldosterone causes a mobility shift of γENaC from an 85 kDa band to 70 kDa band without a change in total γENaC protein abundance[12]. The same changes are observed in chronically sodium-restricted rats in addition to a significant downregulation of the βENaC subunit [12]. Thus, the observed increased apical targeting and altered expression of β- and γENaC subunits in group A could be caused by the stimulation of mineralocorticoid receptor (MR) in the aldosterone-responsive epithelium.

Recent studies demonstrated that the abundance of NCC and αENaC are increased by aldosterone treatment in normal rats[12]. In contrast, in the present study we did not observe any changes of the abundance of NCC and α ENaC in CCl_4-induced liver cirrhosis where plasma aldosterone levels were elevated. Consistent with this, we previously demonstrated that puromycin aminonucleoside (PAN)-induced nephrotic syndrome was associated with an increased apical targeting of ENaC subunits, but the protein abundance of αENaC was not changed in the kidney cortex in the presence of an increased plasma aldosterone levels[7]. Moreover, we previously demonstrated that αENaC abundance was not changed in lithium-treated rats where plasma aldosterone levels were significantly increased[30]. In both PAN and lithium-treated rats, apical trafficking of ENaC subunits was markedly increased while NCC abundances were decreased, suggesting that changes of trafficking of ENaC subunits and abundance of aldosterone sensitive transporters (αENaC and NCC) are dissociated in

pathophysiological conditions. Moreover, mineralocorticoid activity can be regulated by different mechanisms : at the pre-receptor level (11 β HSD2), at the receptor level and at the level of transcriptional activation or repression by cell specific co-factors[31]. Each of these cellular events will ultimately influence the nature and/or the magnitude of the response of the tissue after the stimulation of MR. Thus, it can be speculated that other factors play a role that are at least as effective as aldosterone in modulating ENaC expression/trafficking, or probably the interaction of MR is modified by some local factors in experimental liver cirrhosis.

It has been demonstrated that rats with common bile duct ligation-induced liver cirrhosis had increased natriuretic response to furosemide together with marked hypertrophy of the medullary thick ascending limb (mTAL) cells in the outer medulla[32]. During the later decompensated stage where the renal sympathetic nerves and the renin angiotensin aldosterone system are activated, cirrhosis is associated with avid sodium retention and edema. Concomitant increase of plasma vasopressin, glucagon, and insulin also contribute to the stimulation of sodium rebasorption in the mTAL in cirrhotic rats[32]. We demonstrated the increased abundance of NHE3 and NKCC2 in the outer medulla in CCl_4-treated rats. These observations therefore support the view that the increased renal sodium reabsorption associated with the late decompensated stage of cirrhosis is caused partly by the increased sodium reabsorption in mTAL.

2) Common bile duct ligation (CBDL) **induced biliary liver cirrhosis**

Long-term bile duct ligation in rats is a well known experimental model of liver cirrhosis and portal hypertension, and has been widely used to study the renal mechanisms of sodium retention, as well as systemic and splanchnic hemodynamics[33]. CBDL rats showed decreased fractional excretion of sodium and hence, positive sodium balance in the sodium retaining stage of liver cirrhosis (at 6 wks). It is suggested that increased renal tubular absorption of sodium may account for the sodium retention and pathogenesis of ascites formation. It is noteworthy that at 8 weeks of liver cirrhosis we did not see any evidence of positive sodium balance even though all rats showed marked ascites[34]. These findings may suggest that there are dynamic changes in sodium retention at different stages (i.e., a sodium retaining stage and a compensatory stage) of liver cirrhosis. It should be emphasized that the two time periods are two in a continuum of changes during the development of sodium retention and the stages with partial of full compensation.

The most important finding is the increased targeting of all ENaC subunits to the apical plasma membrane domain in DCT2, CNT and collecting duct in the sodium retaining cirrhotic rats, but not in 8 week liver cirrhotic rats without positive sodium balance. Since increased ENaC plasma membrane targeting is strongly associated with increased sodium reabsorption, this finding very strongly suggests that increased ENaC targetings contribute significantly to the increased renal tubular sodium reabsorption.

Previous studies demonstrate that aldosterone causes a dramatic redistribution of ENaC to the apical plasma membrane[12, 13]. Thus, the increased apical targeting of ENaC subunits in CBDL-induced liver cirrhosis may be caused by increased aldosterone activity via stimulation of mineralocorticoid receptor (MR) in the aldosterone responsive epithelium. Since there was a markedly enhanced apical plasma membrane expression of all ENaC subunits at 6 wk liver cirrhosis associated with maintained plasma aldosterone levels but not at 8 wk liver cirrhosis where aldosterone levels were markedly reduced, it is likely that aldosterone plays an essential role. However, other mechanisms may also contribute as dis-

cussed below.

In vitro studies have demonstrated that the MR has an equal affinity for aldosterone and glucocorticoids, yet in vivo it displays specificity for aldosterone in the face of much higher circulating concentrations of glucocorticoids. This specificity is conferred by 11β HSD2, an enzyme that converts glucocorticoids to inactive 11-ketosteroid derivatives that have weak or no affinity for the MR in mineralocorticoid sensitive tissues including the distal nephron[23]. Loss of function mutation of 11β HSD2 or inhibition of 11β HSD2 activity allows glucocortiocids to promote renal sodium retention and potassium excretion in the cortical collecting tubule[35, 36]. These findings suggest that the 11β HSD2 regulates the sodium reabsorption in the aldosterone responsive renal tubules by glucocorticoid-induced activation of the MR. We demonstrate in the present studies that there is downregulation of 11β HSD2 abundance in the kidney in decompensated liver cirrhosis rats. This finding is in line with recent studies in compensated liver cirrhosis reported by Quattropani et al.[37] and Escher et al[38]. They demonstrated that 11β HSD2 activity in the kidney was reduced in liver cirrhosis following bile duct ligation[37, 38]. These findings together suggest that reduced expression and activity provides basis for access of glucocorticoids to the MR, thereby promoting increased collecting duct sodium reabsorption despite normal plasma levels of aldosterone in rats with experimental liver cirrhosis. This notion was strongly supported by the increased apical targeting of ENaC subunits in rats with glycyrrhizic acid-induced inhibition of 11β HSD2 activity[39].

Plasma aldosterone levels, at least in humans and rats without liver cirrhosis, have been shown to be reduced in conditions with low expression of 11β HSD2[35, 36]. It is noteworthy that plasma aldosterone levels were not reduced in 6 wk liver cirrhosis rats, despite that the protein abundance of 11β HSD2 was significantly decreased. Thus, coordinated activation of MR by both glucocorticoid as a consequence of reduced activity of 11β HSD2 and by relatively high plasma aldosterone may enhance distal renal tubular sodium reabsorption and potassium loss, and hence play important roles for the pathogenesis of sodium retention and possible ascites formation in CBDL-induced liver cirrhosis. In contrast, in response to liver cirrhosis for 8 weeks ENaC abundance was decreased and the reduction was associated with decreased plasma aldosterone levels. In addition, the increased apical targeting of ENaC subunits was retrieved in spite of decreased abundance of 11β HSD2. It can be speculated that 11β HSD2 is still functioning but not at full power (thus aldosterone may play a major role), or it may be speculated that differences in ENaC trafficking between 6 and 8 weeks are independent of aldosterone and/or 11β HSD2.

In normal rats both the apical targeting and protein abundance of αENaC are increased by high dose aldosterone treatment[12]. However, in the present study we did not observe an increase in αENaC protein expression in the cortex in kidneys from rats with CBDL-induced liver cirrhosis for 6 weeks although increased abundance of αENaC was seen in ISOM[34]. It may be speculated that glucocorticoids may be involved in this regulation, and if this is the case the observations suggest that regulation of αENaC abundance is not as sensitive, as is the regulation of changes in the subcellular localization of ENaC, to MR activation by glucocorticoids. Whether the differential regulation of ENaC subunit targeting and expression may be related to differences in the kinetics of MR-ligand interaction remains unknown; aldosterone dissociates more slowly from MR than cortisol does, indicating that the stability of the aldosterone-MR complex is higher than that of the cortisol MR complex[40, 41]. In addition, the MR activity can be regulated by at the level

of transcription activation or repression by cell specific co-factors[31].

It is also interesting to note that the rats with liver cirrhosis apparently exhibited dynamic changes in sodium retention and ENaC trafficking. After 6 weeks, liver cirrhosis animals exhibited sodium retention, potassium loss and decreased urine Na/K ratio indicating increased aldosterone-like activity[34]. These findings can be explained in part by decreased abundance of 11βHSD2, which permits the stimulation of MR by glucocorticoid resulting in increased apical targeting of ENaC subunits. On the other hand, at 8 weeks the rats with liver cirrhosis did not exhibit any changes in urinary sodium and potassium excretion even though they all had marked ascites. The underlying mechanism for the time course changes in circulating aldosterone levels and changes in ENaC subunits trafficking remains undefined. However, it can be speculated that at later stages of liver cirrhosis the rise in extracellular fluid volume and enhanced secretion of atrial natriuretic peptide (ANP) contribute to the decrease in plasma aldosterone levels and, hence, limit ENaC abundance/trafficking since ANP has been known to directly inhibit aldosterone secretion in the adrenal glands through specific ANP receptors in the zona glomerulosa[42].

The results strongly suggests that the marked increase in apical ENaC subunit targeting combined with diminished abundance of 11βHSD2 in the DCT2, connecting tubule and collecting duct is likely to play key roles for the sodium retention associated with CBDL induced liver cirrhosis in rats in the sodium retaining stage (i.e. in this model after 6 weeks of CBDL). The subsequent decrease in the expression of ENaC subunits and the reduced targeting after 8 weeks of CBDL-induced liver cirrhosis may contribute to promote sodium excretion at later stages of liver cirrhosis.

3. Spontaneously hypertensive rats (SHR)

In models of genetic (primary) hypertension, the renal involvement may be presented, at least in part, by inappropriate sodium and water retention[43]. The excessive retention of sodium in SHR may be resulted from either a reduced glomerular filtration rate (GFR)[44] or an enhanced tubular reabsorption[45] or both. The activities of Na,K-ATPase and Na/H exchanger in the proximal tubule was found to be significantly higher in young SHR compared to age matched Wistar-Kyoto rats (WKY)[45] 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.

We examined the changes in protein expression levels of renal sodium transporters, epithelial sodium channel 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[46]. It is noteworthy that the increased abundance of ENaC subunits was more prominent in early stage of 6 wk SHR than late hypertensive stage of 12 wk-SHR, and the Na,K-ATPase immunolabeling in both the proximal tubule and the collecting duct was 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 retention in early stage 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 sodium retaining effect in the collecting duct and proximal tubule is prominent in early stage of SHR and the effect is gradually attenuated in late hypertensive stage of SHR[44]. 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.

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 βENaC and γENaC was consistently increased in both ages of SHR. On the other hand, the change of αENaC was relatively minimal, only mild increase in the ISOM in 6 wk-SHR. The changes in expression fits the previously described pattern seen in response to vasopressin treatment[11], and since vasopressin levels and its responsiveness have been shown to be increased in SHR[47] and the observed changes in expression are consistent with this view. Increased sodium reabsorption in the collecting duct is correlated with the increased protein abundance of ENaC subunits at the site of function, i.e., in the plasma membrane. Thus, even though there is no direct evidence of increased apical trafficking in ENaC subunits, the increased total abundance is very likely to contribute to the increased protein abundance in the plasma membrane as well. Moreover, it cannot be excluded that there may have been transient changes in enhanced ENaC trafficking during the initial stage of SHR, and only increased protein abundance may prevail.

Most studies indicate low or normal plasma aldosterone levels[48, 49], 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[50].

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 extrude 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[51]. Dramatic increases in Na^+/H^+ exchange activity have also been reported in proximal tubule cells of early SHR, but only a modest increase[52] or no changes[53] 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-SHR compared to age-matched WKY. These findings are consistent with the previous results indicating that proximal tubules of early SHR, but not late hypertensive SHR, have increased Na,K-ATPase activity[54]. In the connecting tubule 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 provide 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-SHR, associated with the in-

creased abundance of ENaC subunits. Thus, enhanced sodium reabsorption in the collecting may be activated by coordinated function of ENaC and Na,K-ATPase in 6 wk-SHR rats.

The results demonstrate that the protein abundance of ENaC subunits was increased in DCT2, CNT, and collecting duct segments in both ages of SHR, of which degree was more prominent in 6 wk-SHR compared with that of 12 wk-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 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[46]. These findings suggest the increased protein abundance of ENaC subunits, 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.

Conclusions

Increased abundance and/or apical targeting of ENaC subunits in the DCT2, connecting tubule and collecting duct play an important role in the pathogenesis of sodium retention.

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