Elevated BSC-1 and ROMK Expression in Dahl Salt-Sensitive Rat Kidneys

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Abstract—This study compared the expression of enzymes and transport and channel proteins involved in the regulation of sodium reabsorption in the kidney of Dahl salt-sensitive (DS) and salt-resistant Brown-Norway (BN) and consomic rats (SS.BN13), in which chromosome 13 from the BN rat has been introgressed into the DS genetic background. The expression of the Na⁺/K⁺/2Cl⁻ (BSC-1) cotransporter, Na⁺/H⁺ exchanger (NHE3), and Na⁺-K⁺-ATPase proteins were similar in the renal cortex of DS, BN, and SS.BN13 rats fed either a low-salt (0.1% NaCl) or a high-salt (8% NaCl) diet. The expression of the BSC-1 and the renal outer medullary K⁺ channel (ROMK) were higher, whereas the expression of the cytochrome P450A4 proteins responsible for the formation of 20-hydroxyeicosatetraenoic (20-HETE) was lower in the outer medulla of the kidney of DS than in BN or SS.BN13 rats fed either a low-salt or a high-salt diet. In addition, the renal formation and excretion of 20-HETE was lower in DS than in BN and SS.BN13 rats. These results suggest that overexpression of ROMK and BSC-1 in the thick ascending limb combined with a deficiency in renal formation of 20-HETE may predispose Dahl S rats fed a high-salt diet to Na⁺ retention and hypertension. (Hypertension. 2004; 43:860-865.)

Key Words: rats, Dahl ■ Na⁺, K⁺-transporting ATPase ■ sodium ■ hypertension

The concept that the kidney plays an important role in the long-term control of arterial pressure is based on the pressure–natriuretic response. Guyton et al.1 were the first to recognize that because elevations in arterial pressure directly increase Na⁺ excretion, hypertension can only develop when the pressure–natriuretic relationship is impaired. Renal transplantation studies2 have strongly supported this hypothesis and indicate that some form of renal dysfunction underlies the development of hypertension in man and experimental animals.3

We5,6 and others6,7 have found that the pressure–natriuretic relationship is reset toward higher pressures in Dahl salt-sensitive (DS) rats and that this is caused by an elevation in Cl⁻ reabsorption in the thick ascending limb of the loop of Henle (TALH).8–11 However, the factors that increase Cl⁻ transport in the TALH of DS rats remain to be determined. There is some evidence that a deficiency in the renal formation of 20-hydroxyeicosatetraenoic acid (20-HETE) may be involved. 20-HETE is the primary metabolite of arachidonic acid (AA) produced by the TALH.12,13 It inhibits the activity of the Na⁺/K⁺/2Cl⁻ cotransporter (BSC-1 or NKCC2)12,14,15 and the renal outer medulla K⁺ channel (ROMK)16,17 that maintains the K⁺ gradient for Na⁺/K⁺/2Cl⁻ cotransport in the TALH. The formation of 20-HETE is reduced in the outer medulla of DS rats in comparison to other strains of rats.18–20 Exogenous administration of 20-HETE normalizes Cl⁻ transport in the TALH of DS rats6,9 and induction of the renal formation of 20-HETE which attenuates the development of hypertension.21,22 Finally, blockade of the formation of 20-HETE promotes the development of salt-sensitive hypertension in normally salt-resistant Lewis and Sprague-Dawley rats.8,9

Besides a deficiency in the renal formation of 20-HETE, alterations in the expression of Na⁺/K⁺-ATPase, the BSC-1 cotransporter, and ROMK channels might contribute to the elevation in sodium reabsorption in the TALH of DS rats. Indeed, a molecular variant of the Na⁺/K⁺-ATPase has been linked to altered blood pressure and Na⁺ transport in red blood cells of DS rats.23,24 In addition, Alvarez-Guerra et al.25 have reported that the activity of the BSC-1 is elevated in the TALH of DS rats. This gene maps to a region of rat chromosome 3 that co-segregates with blood pressure in an F2 cross of DS and Lewis rats.26 Mutations in BSC-1 have also been linked to inherited disorders of blood pressure in humans.27 However, previous studies have not examined whether there are differences in the expression of these Na⁺ transport proteins in the kidney of DS versus that seen in salt-resistant strains of rats.

Thus, the present study compared the expression of the α-subunit of Na⁺/K⁺-ATPase, Na⁺/H⁺ exchanger (NHE3), BSC-1, ROMK, and cytochrome P450A4 (CYP4A) proteins...
in the kidney of DS and salt-resistant Brown-Norway (BN) and consomic rats (SS.BN13), in which chromosome 13 from the BN rat has been introgressed into the DS genetic background. The SS.BN13 rat was included as a salt-resistant control strain in this study because it remains normotensive when fed a high-salt diet and is more genetically similar to DS rats (1.9% allelic difference) than any other salt-resistant strain of rat. In this regard, other control strains like Dahl salt-resistant (DR) or BN rats exhibit a 30% and 77% allelic difference to DS across the genome.

Methods

Experimental Animals

Experiments were performed on male DS, BN, and SS.BN13 rats weighing between 275 and 325 g. The BN rats were purchased from Harlan Sprague-Dawley (Indianapolis, Ind). The DS and SS.BN13 rats were obtained from colonies maintained at the Medical College of Wisconsin. All protocols were approved by the Animal Welfare Committee at the Medical College of Wisconsin. The rats were fed a purified diet (AIN 76) purchased from Dyets (Bethlehem, Pa) that contained either 0.1% (low-salt content) or 8% NaCl (high-salt content).

Measurement of Blood Pressure

A micromanethane catheter was implanted into the left femoral artery of the rats using aseptic surgical techniques. After a 4-day recovery period, mean arterial pressure (MAP) was recorded from conscious rats that were maintained on either a low-salt or a high-salt diet for 1 week as previously described.

Immunoblots

Microsomes were prepared from the renal cortex or outer medulla as previously described. Renal cortical (10 μg protein) and outer medullary proteins (25 μg protein) were separated on a 7.5% SDS polyacrylamide gel and transferred to a nitrocellulose membrane. Each membrane was incubated with just one primary antibody raised against a protein of interest, followed by a horseradish peroxidase-coupled secondary antibody and developed using an enhanced chemiluminescent kit (West Pico, Pierce-Rockford, Ill). The membranes were exposed to x-ray film and the intensities of the bands were determined using a digitizing system (Un-Scan It, version 5.0). The relative expression of proteins between strains were determined by normalizing the pixel count of the band of interest in BN and SS.BN13 rats versus the mean intensity observed in the DS samples performed on the same blot. We did not attempt to compare the expression of the various proteins in rats fed a low-salt and a high-salt diet for 1 week, because these samples were performed on different gels and it is difficult to control for the different efficiency of the detection system between blots. The Na+/K+ -ATPase α-subunit antibody was purchased from Upstate Biotechnology (catalog no. 05-369, Lake Placid, NY). The NHE3 antibody was purchased from Chemicon (catalog no. MAB3136; Temecula, Calif). The CYP4A antibody was purchased from Gentest Corporation (catalog no. 210174; Woburn, Mass). Antibodies to the BSC-1 (L320) and the ROMK channel (L567) were obtained from Dr Knepper. The Na+/K+ -ATPase and NHE3 antibodies were used at a dilution of 1:10,000. The BSC-1 and ROMK antibodies were used at a dilution of 1:8000 and the CYP4A antibody was used at a dilution of 1:4000.

Immunohistochemistry

Experiments were also performed to determine whether distribution of the BSC-1, ROMK, and CYP4A proteins were similar in the kidneys of DS, BN, and SS.BN13 rats. The kidneys were collected, frozen in liquid nitrogen, and 5-μm-thick frozen sections were prepared. The frozen sections were fixed with alcohol and acetone, air dried, and incubated with BSC-1, ROMK or CYP4A primary antibodies at dilutions of 1:200, 1:100, or 1:100, respectively. The sections were washed and incubated with a secondary antibody at a dilution of 1:100 for 1 hour. The slides were washed and then counterstained with 0.002% Evans Blue to limit autofluorescence. The sections were viewed with a Nikon fluorescence microscope using a 20× or 60× objective, and objective and digital images were obtained. Overlays of the distribution of the FITC-stained primary antibodies (green) and the native images of the cells (red) were constructed using Meta Morph video imaging software (Universal Imaging, Downingtown, Pa).

Renal Metabolism of Arachidonic Acid

The formation of 20-HETE in renal cortical and outer medullary microsomes was measured by incubating microsomes (0.50 mg protein) with [3H]-AA (0.1 μC/mL, 40 μmol/L) at 37°C for 30 minutes. The reactions were terminated by acidification to pH 3.5, extracted twice with ethyl acetate, and the metabolites were separated by HPLC and monitored using a radioactive flow detector as previously described.

Urinary 20-HETE Excretion

Rats were housed in stainless steel metabolic cages and an overnight sample of urine was collected in glass bottles cooled with dry ice to prevent breakdown of 20-HETE. The concentration of 20-HETE in the samples was determined using a fluorescent HPLC assay as previously described.

Statistics

Mean values ±1SE are presented. Significance of differences between mean values was determined with the use of an ANOVA followed by the Student-Newman-Keuls post hoc test; P<0.05 was considered to be significant.

Results

Blood Pressure

MAP was significantly lower in BN rats (91±1 mm Hg, n=8) than in DS (118±2 mm Hg, n=10) or SS.BN13 (115±2 mm Hg, n=10) rats when the rats were maintained on a low-salt diet. Heart rate was also lower in BN rats (374±8 bpm) than in DS (408±8 bpm) and SS.BN13 (404±5 bpm) rats fed a low-salt diet. MAP increased to 155±5 mm Hg in DS rats fed a high-salt diet for 1 week. However, MAP did not increase significantly in either the BN (119±2 mm Hg) or the SS.BN13 (95±1 mm Hg) rats that were fed a high-salt diet. Heart rate increased to 441±11 bpm in DS rats fed a high-salt diet for 1 week. Heart rate did not increase in BN (396±9 bpm) and SS.BN13 (389±7 bpm) rats fed a high-salt diet.

Expression of Renal Transport and CYP4A Proteins in the Renal Cortex

A comparison of the relative expression of BSC-1, NHE3, Na+/K+ -ATPase, and CYP4A proteins in the renal cortex of DS, BN, and SS.BN13 rats are presented in Figure 1. The expression of NHE3, Na+/K+ -ATPase, or CYP4A proteins in the renal cortex of DS, BN, or SS.BN13 rats maintained on either a low-salt or a high-salt diet was not significantly different. In contrast, the expression of CYP4A protein was 3-fold greater in the renal cortex of BN rats and 2-fold higher in SS.BN13 rats than the levels seen in DS rats fed a low-salt diet. The expression of CYP4A protein also remained higher in the renal cortex of BN rats and SS.BN13 rats than in DS rats after these strains were fed a high-salt diet for 1 week.
Expression of Renal Transport and CYP4A Proteins in the Outer Medulla

A comparison of the expression of BSC-1, ROMK, NHE3, and CYP4A proteins in the outer medulla of DS, BN, and SS.BN13 rats is presented in Figure 2. The expression of BSC-1 protein (Figure 2A) was 2× higher in the outer medulla of DS rats fed a low-salt diet than that seen in BN rats, and it was 9× higher than levels seen in SS.BN13 rats. After 1 week of a high-salt diet, the expression of BSC-1 protein was 9× greater in the outer medulla of DS rats relative to the levels seen in BN rats and was 2× higher than that observed in SS.BN13 rats.

The expression of ROMK protein (Figure 2B) was 9-fold and 2.5-fold greater in the outer medulla of DS rats fed a low-salt diet than the levels observed in BN and SS.BN13 rats. After 1 week on a high-salt diet, the expression of ROMK protein was 4-times higher in the outer medulla of DS rats than the levels seen in BN rats, and it was still 30% higher than the levels observed in SS.BN13 rats.

The expression of NHE3 protein in the outer medulla (Figure 2C) was significantly lower in the outer medulla of DS rats relative to the levels observed in BN and SS.BN13 rats fed either a low-salt or high-salt diet. The expression of the CYP4A protein (Figure 2D) in the outer medulla of DS rats fed either a low-salt or a high-salt diet was 2-fold to 3-fold lower than the corresponding values observed in the outer medulla of BN and SS.BN13 rats.

Immunohistochemistry

Representative examples of the cellular distribution BSC-1, ROMK, and CYP4A proteins seen in the renal cortex and outer medulla of DS, BN, and SS.BN13 rats are presented in Figure 3. The cellular localization of these proteins was similar in DS, BN, and SS.BN13 rats. In the renal cortex, BSC-1 (Figure 3D) and ROMK (Figure 3E) protein were only expressed in a few cortical TALH found in these sections. In the outer medulla, both BSC-1 and ROMK were expressed in the TALH (Figure 3A and B). The expression of both of these proteins was restricted to the apical border of these cells in all of the strains and all of the tubules examined. In contrast, CYP4A protein (Figure 3C and F) was avidly expressed throughout the cytoplasm of the proximal tubules and the...
TALH in the renal cortex and in the TALH in the outer medulla in all three strains.

Renal Formation of 20-HETE
A comparison of the formation of 20-HETE in microsomes prepared from the renal cortex and outer medulla of DS, BN, and SS.BN13 rats is presented in Figure 4. The formation of 20-HETE (Figure 4A) in cortical microsomes of BN and SS.BN13 rats fed a low-salt diet was 61% and 89% higher than that seen in DS rats. Likewise, the formation of 20-HETE remained 79% and 115% higher in the renal cortex of BN and SS.BN13 rats than in DS rats after these strains were fed a high-salt diet for 1 week.

The formation of 20-HETE was 4.5-times and 3-times greater in the outer medulla (Figure 4B) of BN and SS.BN13 rats than in DS rats when the strains were maintained on a low-salt diet. Similarly, 20-HETE formation in the outer medulla was 6-times greater in BN and 5-times greater in SS.BN13 rats than in DS rats when these strains were fed a high-salt diet for 1 week.

20-HETE Excretion
The excretion of 20-HETE (Figure 4C) in BN and SS.BN13 rats fed a low-salt diet was 4- to 5-times higher than the values seen in DS rats. After 1 week on a high-salt diet, the excretion of 20-HETE was still 6- and 4-fold greater in BN and SS.BN13 rats than in DS rats.

Discussion
Previous studies established that DS rats have a higher rate of Cl⁻ absorption in the TALH than DR rats and that this contributes to blunted pressure-natriuresis in this strain. The factors that alter Na⁺ and Cl⁻ transport in the TALH of DS rats, however, remain to be determined. The present study compared the expression of BSC-1, NHE3, Na⁺/K⁺-ATPase, and ROMK protein in the kidney of DS, BN, and SS.BN13 rats to determine whether differences in the expression of these proteins contribute to the elevation in loop Cl⁻ transport in DS rats. The results indicate that the expression of BSC-1 and ROMK are greater in the outer medulla of DS rats than in salt-resistant BN and SS.BN13 rats. The overexpression of
BSC-1 and ROMK protein was restricted to the outer medulla, because we did not detect any significant differences in the expression of these proteins in the renal cortex. Immunohistochemical studies confirmed that the expression of the BSC-1 and ROMK proteins is restricted to the apical border of the TALH in the outer medulla and that the cellular distribution of these proteins is similar in all 3 strains studied. We also found that expression of the CYP4A enzymes responsible for the formation of 20-HETE, an endogenous inhibitor of Na\(^+\) transport in the proximal tubule and TALH, which is reduced in the cortex and outer medulla of DS rats relative to salt-resistant BN and SS.BN13 rats. This protein was avidly expressed throughout the cytoplasm of the proximal tubule and the TALH, and these results are consistent with previous observations that CYP proteins are largely membrane-bound in the endoplasmic reticulum.

The increased expression of BSC-1 and ROMK seen in the apical membranes of the TALH of DS rats has potential significance to the sodium retention seen in this strain, because the coupled activities of the BSC-1 cotransporter and the ROMK channel drive active and passive sodium reabsorption in this portion of the nephron. The majority of active sodium reabsorption in the TALH occurs via the apical BSC-1 cotransporter. The activity of ROMK channels is critical to this process, because this channel recycles K\(^+\) to the tubule lumen, which is necessary to maintain a sufficient concentration of K\(^+\) in the tubular lumen to reabsorb Na\(^+\) via the cotransporter. The recycling of K\(^+\) through the ROMK channel is also the primary determinant of the lumen-positive transepithelial potential that drives passive reabsorption of Na\(^+\) in the TALH via the paracellular pathway. Thus, the elevated expression of BSC-1 and ROMK proteins in the apical membrane of the TALH of DS rats would be expected to enhance both active and passive NaCl transport. These findings are consistent with previous observations that the activity of BSC-1 is increased in the TALH of DS rats, and that Cl\(^-\) transport is elevated in the TALH of DS rats perfused in vivo or in vitro. It is also consistent with the observation that lumen-positive transepithelial potential is elevated in the TALH of DS rats perfused in vitro and that the pressure-natriuretic response is blunted in DS rats.

Because 20-HETE is known to inhibit the activity of the BSC-1 cotransporter and the ROMK channels, we compared the expression of CYP4A protein, the renal formation of 20-HETE, and the urinary excretion of 20-HETE in DS, BN, and SS.BN13 rats. The expression of CYP4A protein and the formation of 20-HETE were lower in microsomes prepared from the renal cortex and outer medulla of DS rats relative to BN and SS.BN13 rats, regardless of whether the rats were fed a low-salt or high-salt diet. Furthermore, the excretion of 20-HETE was markedly reduced in DS rats in comparison to BN and SS.BN13 rats. These results further support the view that a deficiency in the renal formation of 20-HETE contributes to the elevated NaCl reabsorption in the TALH of DS rats. Diminished 20-HETE formation, coupled with elevated expression of BSC-1 and ROMK proteins in the TALH, should synergistically interact and predispose DS rats to retain sodium when fed a high-salt diet.

In the present study, we also found that transfer of chromosome 13 from the BN rat into the DS genetic background in SS.BN13 consomic rats reduced the expression of BSC-1 and ROMK protein, increased the expression of CYP4A in the kidney, and increased the renal formation and excretion of 20-HETE. Because the genetic backgrounds of DS and SS.BN13 are nearly identical (<1.9% difference across the entire genome), these findings support the view that substitution of chromosome 13 probably confers protection from salt-induced hypertension by reducing loop Cl\(^-\) transport and improving pressure-natriuresis, in part by normalizing the expression of BSC-1, ROMK, and CYP4A protein in the TALH.

The genes for BSC-1, ROMK, and CYP4A are not located on chromosome 13. Thus, the reason for why substitution of chromosome 13 normalizes the expression of these proteins in SS.BN13 rats remains to be determined. One possibility is that the change in the expression of these proteins might be secondary to a reduction in blood pressure and renal damage. Although a reduction in tubular damage might explain an increase in the expression of CYP4A protein in the kidney of SS.BN13 rats, it is not consistent with the downregulation of the expression of BSC-1 and ROMK proteins seen in SS.BN13 rats. Also arguing against this view is our finding that the differences in the expression of these proteins were still apparent when the DS and SS.BN13 rats were maintained on a low-salt (0.1% NaCl) diet that minimizes differences in blood pressure and renal damage between the strains. Thus, these findings suggest that the differences in the expression of the transport proteins detected in DS relative to SS.BN13 rats may be caused by an interaction between a gene on chromosome 13 that alters the expression of BSC-1, ROMK, and CYP4A to promote Na\(^+\) excretion and to minimize the rise in blood pressure in SS.BN13 rats fed a high-salt diet. The gene or group of genes on chromosome 13 that is responsible for normalizing arterial pressure and the expression of BSC-1, ROMK, and CYP4A remains to be determined. The renin gene is localized on chromosome 13, and there is recent evidence that a failure to suppress the activity of the renin angiotensin system may contribute to the development of hypertension and renal end organ damage in Dahl S rats fed a high-salt diet. Angiotensin II influences the expression of CYP4A and transport proteins in the kidney. Thus, the renin gene remains a viable candidate that may contribute to the differences in the expression of BSC-1, ROMK, and CYP4A proteins in the kidneys of DS and SS.BN13 rats. We are currently conducting backcross and F\(_2\) mapping studies to narrow the interval on chromosome 13 to better-identify the genes responsible for the changes in blood pressure and the expression of BSC-1, ROMK, and CYP4A protein in the SS.BN13 rats.

**Perspectives**

The present results indicate that BSC-1 and ROMK proteins are overexpressed in the TALH of DS rats and that CYP4A protein levels and the renal formation and excretion of 20-HETE are significantly lower in DS rats than in salt-resistant BN and SS.BN13 rats. These results suggest that overexpression of BSC-1 and ROMK coupled with a dimin-
ished formation of 20-HETE may predispose DS rats to sodium retention and hypertension when challenged with a high-salt diet.

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