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Original Paper

Alteration of Tight Junction Protein Expression in Dahl Salt-Sensitive Rat Kidney

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Key Words
Claudin-4 • Dahl salt-sensitive rats • Hypertension • Occludin • Pressure natriuresis

Abstract
Background/Aims: Altered pressure natriuresis is an important mechanism of hypertension, but it remains elusive at the molecular level. We hypothesized that in the kidney, tight junctions (TJs) may have a role in pressure natriuresis because paracellular NaCl transport affects interstitial hydrostatic pressure. ***Methods:*** To assess the association of salt-sensitive hypertension with altered renal TJ protein expression, Dahl salt-sensitive (SS) and salt-resistant (SR) rats were put on an 8% NaCl-containing rodent diet for 4 weeks. Systolic blood pressure (SBP) and urine NaCl excretion were measured weekly, and kidneys were harvested for immunoblotting and quantitative PCR analysis at the end of the animal experiments. ***Results:*** SBP was significantly higher in SS rats than in SR rats during the first to fourth weeks of the animal experiments. During the first and second week, urinary NaCl excretion was significantly lower in SS rats as compared with SR rats. However, the difference between the two groups vanished at the third and fourth weeks. In the kidney, claudin-4 protein and mRNA were significantly increased in SS rats as compared with SR rats. On the other hand, occludin protein and mRNA were significantly decreased in SS rats as compared with SR rats. The expression of claudin-2, claudin-7, and claudin-8 did not vary significantly between the two groups. ***Conclusions:*** In SS rats, SS hypertension was associated with differential changes in renal TJ protein expression. Both upregulation of claudin-4 and downregulation of occludin might increase paracellular NaCl transport in the kidney, resulting in impaired pressure natriuresis in SS rats.

Introduction

The kidney plays a pivotal role in the pathogenesis of hypertension because its excretion of sodium determines both body fluid volume and peripheral vascular resistance [1]. In pressure natriuresis, arterial pressure is maintained over a narrow range as the kidneys

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adjust to natriuresis. When the normal relationship of pressure natriuresis is right-shifted, abnormal hypertension is induced [2]. Thus, increased renal tubular sodium reabsorption is one of the major causes of chronic hypertension. The pathways of sodium transport through the renal tubular epithelia are both transcellular and paracellular. Previous studies on mechanisms of pressure natriuresis have focused on the regulation of transcellular sodium transporters in the kidney [3, 4]. The results suggest that proximal tubule sodium transporters are not primarily involved [5], but have a compensatory action in response to changes in extracellular fluid volume and blood pressure.

Paracellular NaCl transport through the tight junction (TJ) occurs along the nephron, and the TJ includes integral membrane proteins such as claudins and occludin, scaffolding proteins such as zonula occludens-1 (ZO-1), and signaling proteins from all major families of G proteins, kinases, and phosphatases [6]. In addition to active sodium transport, passive diffusion of NaCl through paracellular pathways contributes to renal interstitial hydrostatic pressure (RIHP) and may affect pressure natriuresis [7]. Ramsey et al. demonstrated that increased RIHP enhances paracellular backflux from the renal interstitium to the tubular lumen through the intercellular TJs [8].

Claudins are TJ proteins that regulate the paracellular permeability of the renal epithelia along the nephron segments [9], and different claudins act as paracellular pores and barriers [10]. A previous study demonstrated that collecting duct-specific claudin-4 knockout animals develop hypotension due to profound renal wasting of chloride [11]. Besides, altered immunoreactivity for occludin and ZO-1 was reported in rats with hypertension [12]. We hypothesized that TJ proteins may have a role in pressure natriuresis or salt-sensitive (SS) hypertension because paracellular NaCl transport affects interstitial hydrostatic pressure in the kidney. To test this, we used the Dahl SS rat model of hypertension in which SS hypertension has been induced and the pressure-natriuresis curve has been shifted to the right compared with Dahl salt-resistant (SR) rats [13].

Materials and Methods

Animal experiments

Male 5-week-old Dahl SS (n=6) and SR (n=6) rats weighing 180-200 g were purchased (SLC Inc., Shizuoka, Japan). After acclimatization for a week, all rats were fed a rodent diet with 8.0% NaCl (DYET #100078 AIN-76A Purified Rodent Diet with 8.0% NaCl, Dyets Inc., Bethlehem, PA) for four weeks. They could freely access drinking water and were housed weekly in metabolic cages for urine collection. Systolic blood pressure was monitored weekly using a tail-cuff method (P-98A, Softron, Tokyo, Japan). At the same time, blood samples were also collected from the tail veins. At the end of the animal experiments, kidneys were harvested to perform semi-quantitative immunoblotting and quantitative polymerase chain reaction (qPCR) analyses for TJ proteins and mRNA transcripts, respectively. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Hanyang University (*HY-IACUC-15-0003*).

Immunoblot analysis

Manually dissected slices of whole kidney were homogenized in a buffer containing 250 mM sucrose, 10 mM triethanolamine, 1 μg/mL leupeptin, and 0.1 mg/mL phenylmethylsulfonyl fluoride titrated to pH 7.6. Coomassie-stained "loading gels" were prepared in order to assess the accuracy of protein loading before immunoblotting [14]. Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 8% gels for ZO-1 and E-cadherin, 10% gels for occludin, and 12% gels for claudin-2, claudin-4, claudin-7, and GAPDH. For immunoblotting, the proteins were transferred electrophoretically from unstained gels to nitrocellulose membranes (Bio-Rad, Hercules, CA). After blocking with 5% skim milk in PBS-T (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, 0.1% Tween-20, pH 7.5) for 1 hr, the membranes were probed overnight at 4°C with the respective primary antibodies: rabbit polyclonal anti-occludin (Thermo Fisher, Rockford, IL), rabbit polyclonal anti-ZO-1, mouse monoclonal anti-claudin-2, rabbit polyclonal anti-claudin-4, mouse monoclonal anti-claudin-7 (Invitrogen, Carlsbad,

Table 1. Primer sequences for qPCR. qPCR, quantitative polymerase chain reaction; ZO-1, zonula occludens-1

Gene	Forward (F) and reverse (R) primer sequences	PCR product (bp)	GenBank Accession No.
Occludin	F 5′-CTGTCTATGCTCGTCATCG-3′ R 5′-CATTCCCGATCTAATGACGC-3′	284	NM-031329
ZO-1	F 5′-AGCGAAGCCACCTGAAGATA-3′ R 5′-GATGGCCAGCAGGAATATGT-3′	139	NM-001106266
Claudin-2	F 5′-TCTGGATGGAGTGTGCGAC-3′ R 5′-AGTGGCAAGAGGCTGGGC-3′	466	NM-001106846
Claudin-4	F 5′-CGAGCCCTGATGGTCATCAG-3′ R 5′-CGGAGTACTTGGCGGAGTAG-3′	358	NM-001012022
Claudin-7	F 5′-TGGCAGGTCTTGCTGCTTTG-3′ R 5′-TGCCCAGCCGATAAAGATGG-3′	128	NM-031702
Claudin-8	F 5′-GGTGAAGCCCTCTACATAGG-3′ R 5′-CGTGGAAACTCCTCTGAGTG-3′	148	NM-001037774

CA), mouse monoclonal anti-E-cadherin (BD Biosciences, Franklin Lakes, NJ), and rabbit monoclonal anti-human GAPDH (Cell Signaling Technology, Beverly, MA). The secondary antibody was goat anti-rabbit IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA). The sites of antibody-antigen reaction were viewed using enhanced chemiluminescence (GenDEPOT, Barker, TX), and the band densities on the immunoblots were quantified by densitometry using a laser scanner and Quantity One software (Basic version 4.6.9, Bio-Rad).

qPCR analysis

Total RNA was isolated from rat whole kidney with TRIzol® Reagent (Life Technologies, Carlsbad, CA). RNA was quantified by spectrophotometry, and cDNA synthesis was performed on 3 μg of RNA with SuperScript® III Reverse Transcriptase (Life Technologies). For qPCR, 100 ng of cDNA served as a template for PCR amplification using the Brilliant SYBR green QPCR master mix, according to the manufacturer's instructions (FastStart DNA Master SYBR Green I; Roche Molecular Biochemicals, Mannheim, Germany). Serial dilution (1 ng/μL - 1 fg/μL) of cDNA was used as a template to generate a standard curve. Nested primers were used to amplify the standard and the kidney cDNA samples (Table 1). The standard and unknown samples were amplified in duplicate in 96-well plates. The thermal profile of the LightCycler® Instrument (Roche Molecular Biochemicals) was optimized with an initial denaturation for 10 minutes at 95°C, followed by 45 amplification cycles, each consisting of 10 seconds at 95°C, 10 seconds at 60°C, and 10 seconds at 72°C. The comparative Ct method was used to determine the relative amounts of target-mRNA transcripts, expressed for each sample as a percentage of GAPDH mRNA levels. Ct ratios were analyzed using the LightCycler® Software (Version 4.05). Specificity was verified by post-run melting-curve analysis [15].

Statistics

Values are presented as mean ± SE. Quantitative comparisons between groups were performed using the Mann-Whitney *U*-test (Statview software; Abacus Concepts, Berkeley, CA). To facilitate immunoblot and qPCR comparisons, we normalized the band density or the relative mRNA values by dividing them by the mean value for the SR group. Thus the mean for the SR group was defined as 100%. $P < 0.05$ was considered to indicate statistical significance.

Results

Table 2 presents results from the 7th, 14th, 21st and 28th day of the animal experiments. Body weight did not vary significantly between groups during the first half of this period, but weight gain was significantly lower in SS rats than in SR rats during the second half of the animal experiments. In contrast, urine volume was significantly lower in SS rats than in SR rats during the first half of this period, but was not different between groups during the second half of the animal experiments. Consistent with this, urinary sodium and chloride

Table 2. Functional parameters in the animal experiment. Values are mean ± SE. SR, Dahl salt-resistant rats; SS, Dahl salt-sensitive rats; BUN, blood urea nitrogen; BW, body weight; NS, not significant. P<0.05 vs. SR by Mann-Whitney U test

Parameters	SR (n=6)	SS (n=6)	P
7th day			
Body weight (g)	236 ± 2	240 ± 2	NS
BUN (mg/dL)	16.4 ± 1.1	13.8 ± 0.8	NS
Plasma sodium (mmol/L)	137 ± 1	137 ± 2	NS
Plasma chloride (mmol/L)	104 ± 1	101 ± 2	NS
Plasma creatinine (mg/dL)	0.12 ± 0.01	0.10 ± 0.02	NS
Urine output (mL/d/100 g BW)	41.1 ± 0.6	34.4 ± 1.2	<0.05
Urine sodium excretion (mmol/d/100 g BW)	14.0 ± 0.2	12.3 ± 0.3	<0.05
Urine chloride excretion (mmol/d/100 g BW)	13.7 ± 0.1	11.9 ± 0.3	<0.05
Urine potassium excretion (mmol/d/100 g BW)	0.63 ± 0.03	0.65 ± 0.02	NS
Creatinine clearance (mL/min/100 g BW)	2.1 ± 0.1	3.1 ± 0.8	NS
14th day			
Body weight (g)	289 ± 2	283 ± 3	NS
BUN (mg/dL)	17.2 ± 1.5	19.4 ± 1.2	NS
Plasma sodium (mmol/L)	143 ± 1	139 ± 0	<0.05
Plasma chloride (mmol /L)	105 ± 2	101 ± 1	<0.05
Plasma creatinine (mg/dL)	0.13 ± 0.05	0.14 ± 0.04	NS
Urine output (mL/d/100 g BW)	30.5 ± 0.6	25.3 ± 1.4	<0.05
Urine sodium excretion (mmol/d/100 g BW)	10.3 ± 0.1	9.4 ± 0.3	<0.05
Urine chloride excretion (mmol/d/100 g BW)	10.0 ± 0.1	9.1 ± 0.3	<0.05
Urine potassium excretion (mmol/d/100 g BW)	0.46 ± 0.01	0.53 ± 0.01	<0.05
Creatinine clearance (mL/min/100 g BW)	2.4 ± 0.7	1.7 ± 0.2	NS
21st day			
Body weight (g)	336 ± 2	315 ± 4	<0.05
BUN (mg/dL)	14.1 ± 1.0	14.6 ± 1.0	NS
Plasma sodium (mmol/L)	140 ± 1	138 ± 1	NS
Plasma chloride (mmol/L)	107 ± 2	103 ± 1	NS
Plasma creatinine (mg/dL)	0.09 ± 0.02	0.09 ± 0.02	NS
Urine output (mL/d/100 g BW)	23.9 ± 0.6	24.6 ± 1.0	NS
Urine sodium excretion (mmol/d/100 g BW)	8.6 ± 0.1	9.2 ± 0.2	<0.05
Urine chloride excretion (mmol/d/100 g BW)	8.4 ± 0.1	8.9 ± 0.2	NS
Urine potassium excretion (mmol/d/100 g BW)	0.55 ± 0.01	0.59 ± 0.01	<0.05
Creatinine clearance (mL/min/100 g BW)	6.8 ± 4.2	3.5 ± 0.9	NS
28th day			
Body weight (g)	351 ± 3	327 ± 4	<0.05
BUN (mg/dL)	23.0 ± 2.6	20.8 ± 1.1	NS
Plasma sodium (mmol/L)	135 ± 2	136 ± 1	NS
Plasma chloride (mmol/L)	103 ± 2	103 ± 1	NS
Plasma creatinine (mg/dL)	0.20 ± 0.01	0.18 ± 0.01	NS
Urine output (mL/d/100 g BW)	22.6 ± 0.3	23.8 ± 1.2	NS
Urine sodium excretion (mmol/d/100 g BW)	8.3 ± 0.2	9.0 ± 0.3	NS
Urine chloride excretion (mmol/d/100 g BW)	7.9 ± 0.2	8.7 ± 0.3	NS
Urine potassium excretion (mmol/d/100 g BW)	0.48 ± 0.03	0.51 ± 0.02	NS
Creatinine clearance (mL/min/100 g BW)	1.2 ± 0.1	1.5 ± 0.1	NS

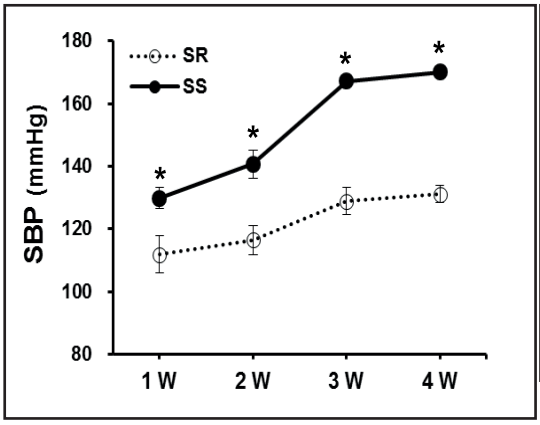

Fig. 1. Changes in systolic blood pressure from Dahl salt-sensitive and salt-resistant rats given an 8% NaCl-containing rodent diet. Data are means ± standard errors. SS (•), salt-sensitive rats (n=6); SR (o), salt-resistant rats (n=6); W, week. *P<0.05 by the Mann-Whitney U-test.

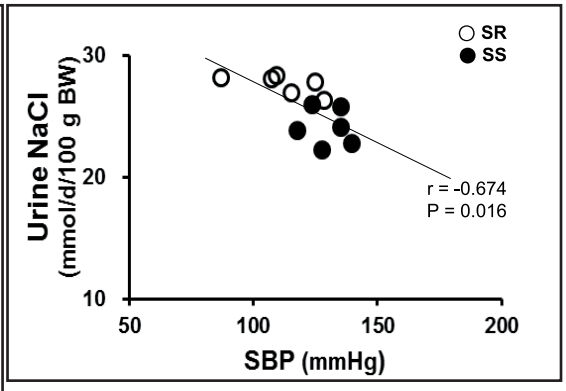

Fig. 2. The relationship between systolic blood pressure (SBP) and urinary excretion of NaCl from Dahl salt-sensitive and salt-resistant rats given an 8% NaCl-containing rodent diet. Data were obtained from the 1st week of the animal experiment. SS (•), salt-sensitive rats (n=6); SR (o), salt-resistant rats (n=6).

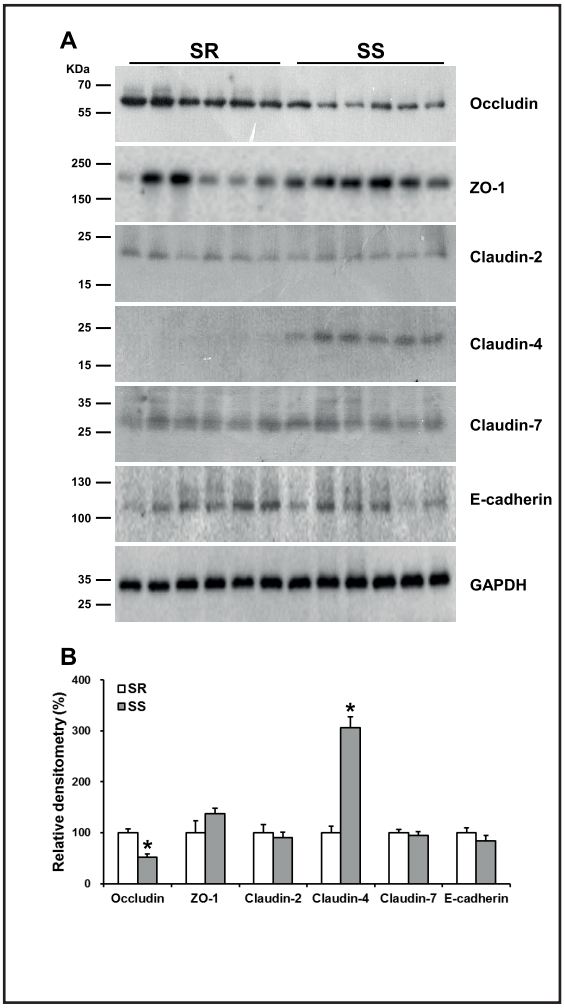

Fig. 3. Immunoblots of tight junction proteins in kidneys from Dahl salt-sensitive and salt-resistant rats given an 8% NaCl-containing rodent diet. Kidneys were harvested after 4 weeks of animal experiments. (A) Immunoblots, in which each lane was loaded with a protein sample from a different rat and reacted with a specific antibody, are shown. (B) Densitometric analyses revealed a significant decrease in occludin and a significant increase in claudin-4 expression in Dahl SS versus SR rats. Data are means ± standard errors. SS, salt-sensitive rats; SR, salt-resistant rats. *P<0.05 by the Mann-Whitney U-test.

excretion was significantly lower in SS rats than in SR rats at the end of first and second weeks. In contrast, urinary potassium excretion was significantly higher in SS rats than in SR rats at the end of the second and third weeks. Blood urea nitrogen and plasma creatinine concentration were not significantly different between groups throughout the animal experiments (Table 2).

As expected, systolic blood pressure (SBP) was significantly higher in SS rats than in SR rats throughout the animal experiments (Fig. 1). When urinary NaCl excretion was plotted against SBP, an increased SBP was associated with impaired NaCl excretion at the first week (r = - 0.674, *P*<0.05; Fig. 2).

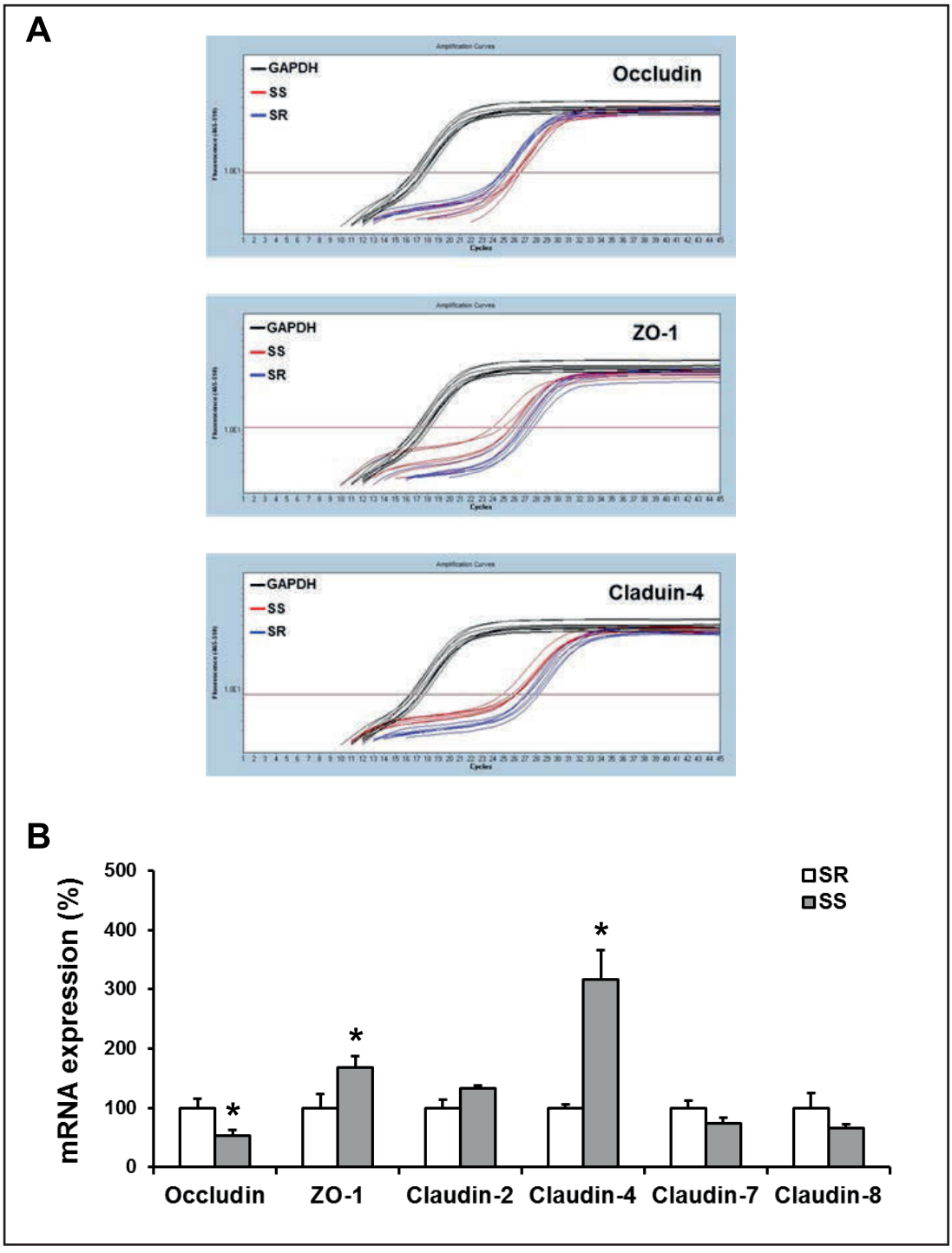

Fig. 4. Quantitative PCR data for mRNA levels of tight junction proteins in kidneys from Dahl salt-sensitive and salt-resistant rats given an 8% NaCl-containing rodent diet. Kidneys were harvested after 4 weeks of animal experiments. (A) Occludin, ZO-1, and claudin-4 graphs of fluorescence plotted against cycle number discriminated between Dahl SS and SR rats. Horizontal bars represent the threshold cycle. (B) Compared with Dahl SR rat kidneys, Dahl SS rat kidneys had a lower level of occludin mRNA and significantly higher mRNA levels of ZO-1 and claudin-4. Data are means ± standard errors. SS, salt-sensitive rats; SR, salt-resistant rats. *P<0.05 by the Mann-Whitney U-test.

Fig 3 shows Immunoblots of TJ proteins in whole kidneys from SS and SR rats. Immunoblot analysis revealed that occludin protein abundance was significantly decreased in SS rats compared with SR rats (52 ± 16% *vs.* 100 ± 19%, P<0.05). On the other hand, the abundance of claudin-4 protein was significantly increased in SS rats compared with SR rats (306 ± 52% *vs.* 100 ± 31%, P<0.05). However, the protein abundance of ZO-1, E-cadherin, claudin-2, and claudin-7 was not significantly different between groups.

The results of the qPCR analysis for TJ mRNA transcripts in whole kidneys are summarized in Fig. 4. Graphs of fluorescence plotted against cycle number discriminated between SS and SR rats. Consistent with the results of the semiquantitative immunoblotting, SS rats had a lower mRNA expression level of occludin (52 ± 10% *vs.* 100 ± 16%, *P*<0.05) and a higher mRNA expression level of claudin-4 (316 ± 50% *vs.* 100 ± 6%, *P*<0.05) compared with SR rats. Interestingly, the mRNA level of ZO-1 was significantly higher in SS rats than in SR rats (168 ± 19% *vs.* 100 ± 23%, *P*<0.05). However, mRNA levels of claudin-2, claudin-7, and claudin-8 were not significantly different between groups.

Discussion

In this study, we suggested a pathophysiological role of renal TJ proteins in SS hypertension. In Dahl SS rats, SS hypertension was associated with differential changes in renal TJ protein expression. Both upregulation of claudin-4 and downregulation of occludin might increase paracellular NaCl transport in the kidney, resulting in impaired pressure natriuresis in SS rats (Fig. 5). The upregulation of claudin-4 might oppose RIHP because of increasing paracellular chloride transport [11]. Downregulation of occludin would reduce its barrier function [16] and lessen the effect of pressure natriuresis.

The different blood pressure responses to high salt intake in Dahl SS versus SR rats are inherited; the kidney has been the focus of research on the mechanisms of salt-sensitive hypertension because the ability of Dahl SS rats to excrete a sodium load is significantly lower than that in SR rats [17]. Previous studies have shown that the natriuretic response to increased renal interstitial hydrostatic pressure is reduced in Dahl SS rats compared with Dahl SR rats [18, 19]. We also demonstrated that urinary NaCl excretion was reduced in Dahl SS rats with hypertension. Because the glomerular filtration rate estimated by creatinine

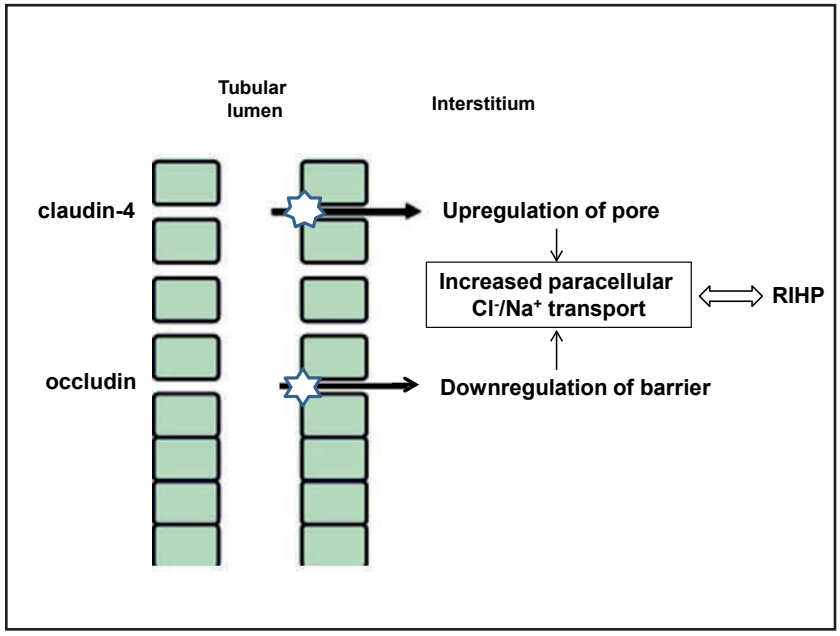

Fig. 5. Postulated contribution of altered tight junction proteins to impaired pressure natriuresis. Upregulation of claudin-4 would increase paracellular Cl^- transport, and downregulation of occludin would reduce barrier function and increase paracellular Na^+ transport. Both processes would oppose renal interstitial hydrostatic pressure (RIHP) and lessen the effect of pressure natriuresis.

clearance was not different between the SS and SR rats, renal tubular reabsorption of NaCl must have increased in the SS rats.

Previous studies focusing on the role of transcellular sodium transporters have not provided a consistent explanation. A mutation in the α1 Na/K-ATPase gene, leading to higher sodium reabsorption and SS hypertension, was identified in Dahl SS rats [20, 21], but its existence was later denied by other researchers [22]. The contributory role of the proximal tubule Na^+/H^+ exchanger type 3 was also unclear in Dahl SS rat kidneys [23, 24]. Although Hoagland et al. reported that the thick ascending limb Na^+-K^+-$2Cl^-$ cotransporter was upregulated in Dahl SS rat kidneys, the control animals used were not Dahl SR rats, but rather salt-resistant brown Norway rats [25]. According to Kakizoe et al., the expression of epithelial sodium channels was increased in Dahl SS rat kidneys compared with SR rat kidneys [26].

Paracellular pathways through the TJs are the alternative routes of NaCl transport along the nephron. Among TJ proteins, claudins regulate the paracellular permeability of the renal epithelia, and we tested whether the expression of claudin-2, -4 -7, or -8 was altered in our animal model of SS hypertension. Claudin-2 has a role in Na^+ reabsorption in the proximal tubule [10], but its expression was not associated with impaired natriuresis in our Dahl SS rats. On the other hand, the expression of claudin-4, recently reported to act as an important route for Cl^- reabsorption in the collecting duct [27], was increased at both the protein and mRNA level in our animal model of SS hypertension. This finding is consistent with a previous study demonstrating that chloride contributes to the development and severity of hypertension in the Dahl SS rat [28].

Claudin-7 and -8 are also expressed in the collecting duct [27]. Although severe renal salt wasting and hypovolemia was induced by genetic ablation of claudin-7 in mice [29], it is not clear whether claudin-7 directly contributes to the paracellular chloride pathway [27]. In this study, the renal expression of claudin-7 was not associated with impaired natriuresis in our Dahl SS rats. Claudin-8 is another candidate to have a role in paracellular chloride transport in the collecting duct [30], but its mRNA level was not changed in our animal model of SS hypertension.

We found that the renal expression of occludin was decreased in our animal model of salt-sensitive hypertension. Occludin is an important molecule, responsible for determination of the paracellular barrier [31]. Raikwar et al. have shown that Nedd4-2 ubiquitinates occludin in the collecting duct epithelia, leading to decreased occludin levels and enhanced paracellular conductance [32]. These results suggest a role of occludin in the paracellular sodium transport along the connecting tubule and collecting duct because Nedd4–2 plays a central role in the regulation of aldosterone-stimulated, amiloride-sensitive sodium transport in the kidney [33]. We postulate that downregulation of occludin in our Dahl SS rat kidneys would enhance paracellular conductance in the connecting tubule and collecting duct, resulting in impaired natriuresis.

Last, the renal mRNA expression of ZO-1 was increased in our animal model of SS hypertension. However, we found no changes in the protein level. These discrepant results may be related to the fact that ZO-1 is located exclusively at the TJ and links TJ proteins to each other [6]. Whether ZO-1 has a role in paracellular NaCl transport in the kidney needs to be answered in the future.

We conclude that in Dahl SS rats, SS hypertension was associated with differential changes in renal TJ protein expression. Both upregulation of claudin-4 and downregulation of occludin might increase paracellular NaCl transport in the kidney, resulting in impaired pressure natriuresis in SS rats.

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Disclosure Statement

None declared.

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