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Original Article

Herbal medicine (Oryeongsan) for fluid and sodium balance in renal cortex of spontaneously hypertensive rats

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ABSTRACT

Background: Herbal medicine Oryeongsan (ORS), also known as Wulingsan in Chinesehas been used for the treatment of impaired body fluid balance. However, the mechanisms involved are not clearly defined. The purpose of the present study was to identify the actions of ORS on the renal excretory function and blood pressure (BP) and to define the mechanisms involved in association with renin-angiotensin system (RAS) and natriuretic peptide system (NPS) in spontaneously hypertensive rats (SHR), an animal model of human essential hypertension.

Methods: Changes in urine volume (UV), excretion of electrolytes including Na^+ (urinary excretion of Na^+ ($U_{Na}V$)) were measured. RT-PCR was performed to trace the changes in expression of RAS, NPS and sodium (Na^+)-hydrogen (H^+) exchanger 3 (NHE3) in the renal cortex.

Results: In the SHR treated with vehicle (SHR-V) group, UV and $U_{Na}V$ were suppressed and the Na^+ balance was maintained at the higher levels leading to an increase in BP compared to WKY-V group. These were accompanied by an increase in NHE3 expression with an accentuation of angiotensin I converting enzyme-angiotensin II type 1 (ACE-AT_1) receptor and concurrent suppression of angiotensin II type 2 (AT_2) receptor/ACE2-Mas receptor expression in the renal cortex. Chronic treatment with ORS increased UV and $U_{Na}V$, and decreased the Na^+ and water balance with a decrease in BP in the ORS-treated SHR-ORS group compared to SHR-V. These were accompanied by a decrease in NHE3 expression with a suppression of ACE-AT_1 receptor and concurrent accentuation of AT_2/ACE2-Mas receptor.

Conclusion: The present study shows that ORS reduced BP with a decrease in Na^+ and water retention by a suppression of NHE3 expression via modulation of RAS and NPS in SHR. The present study provides pharmacological rationale for the treatment of hypertension with ORS in SHR.

1. Introduction

Herbal medicine Oryeongsan (ORS) has long been used for the treatment of impaired body fluid homeostasis. Earlier on, ORS was found to increase urinary volume (UV), salt excretion including sodium (Na^+) and glomerular filtration rate (GFR) in anesthetized rabbits.[1] Later, it was shown that subacute treatment with ORS suppressed plasma levels of renin activity and aldosterone with an increase in GFR, UV and urinary excretion of Na^+ ($U_{Na}V$) in Sprague-Dawley rats.[2] Recently, it was further shown that chronic treatment with ORS accentuated atrial natriuretic peptide (ANP) secretion in the atria from spontaneously hypertensive rats (SHR) in which the hormone secretion was suppressed.[3] The ORS accentuation for the ANP secretion in the atria from SHR was accompanied by suppression of angiotensin (Ang) I converting enzyme (ACE)-Ang II type 1 (AT_1) receptor signaling and accentuation of AT_2 receptor/ACE2-Mas receptor signaling pathway expression in the

Abbreviations: ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; ACh, acetylcholine; ANP, atrial natriuretic peptide; AT_1 receptor, angiotensin II type 1 receptor; AT_2 receptor, angiotensin II type 2 receptor; FENa%, per cent changes in fractional excretion of Na^+; GFR, glomerular filtration rate; NHE3, Na^+-H^+-exchanger-3; NPS, natriuretic peptide system; ORS, oryeongsan; PRA, plasma renin activity; RAS, renin-angiotensin system; SBP, systolic blood pressure; SHR, spontaneously hypertensive rats; $U_{Cl}V$, urinary excretion of chloride; U_KV, urinary excretion of potassium; $U_{Na}V$, urinary excretion of sodium; UV, urinary flow (volume).

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atria. These findings are in relation with the notion that the herbal formula ORS may promote the cardio-renal function leading to volume and pressure homeostasis via modulation of the renin-angiotensin system (RAS) and natriuretic peptide system (NPS). The herbal formula ORS originates from the traditional Chinese medicine book, "Treatise on Febrile Disease" (Shanghanlun in Chinese), written by Zhang ZhongJing in the third century. The formula ORS is composed of 5 medicinal herbs, *Alisma orientale* Juzepzuk, *Poria cocos* Wolf, *Atractylodes macrocephala* Koidzumi, *Polyporus umbellatus* Fries, and *Cinnamomum cassia* Presl, in the ratio of 5:3:3:3:1 in weight, respectively.[2,4,5]

The beneficial effects of the treatment with ORS in the Goldblatt model of renovascular (Goldblatt) hypertensive rats, promotion of the renal function including natriuresis, diuresis, and decrease in systolic blood pressure (SBP), were accompanied by modulation of the RAS and NPS.[6] Chronic treatment with ORS decreased abundance of Na^+/H^+ exchanger 3 (NHE3) gene expression along with a suppression of ACE-AT_1 receptor and concurrent accentuation of AT_2 receptor/ACE2-Mas receptor pathway in the cortex of the kidney from Goldblatt hypertensive rats. It was also shown that chronic treatment with ORS powder decreased SBP in Goldblatt hypertensive rats and SHR[7,8] with a decrease in plasma levels of renin activity (PRA), Ang II, aldosterone, and a decrease in AT_1 receptor gene expression and an increase in ACE2 expression in the ventricular myocardium.[8] Because the long-term regulation of blood pressure is based on the body fluid and Na^+ metabolism through the infinite gain of the renal responses via modulation of the RAS and NPS signaling,[9–11] it is reasonable to hypothesize that ORS participates in blood pressure control through the regulation of the body fluid and salt balance homeostasis. The purpose of the present study was to determine the effects of ORS on the salt and water metabolism in association with the changes of the RAS and NPS in the renal cortex of SHR, an experimental model for human essential hypertension, and to identify the mechanisms involved. Recently, it was known that the AT_2 receptor-NHE3 signaling is defective in SHR but not WKY.[12–14] The RAS has crucial roles in the regulation of salt and water balance and blood pressure homeostasis. The ACE-AT_1 receptor signaling pathway is for the Na^+ retention, vasoconstriction, and pro-hypertensive, and Ang III-AT_2 receptor and ACE2-Mas receptor signaling are for the natriuresis, vasodilation, and anti-hypertensive.[15–18] Furthermore, NHE3 located at the renal proximal tubule is involved in the regulation of Na^+ reabsorption in major proportion of the glomerular filtrate under the control of the ACE-Ang II-AT_1 receptor and Ang III-AT_2 receptor signaling,[12,17] and in the regulation of SBP.[17,19] In addition, the NPS is considered as a counter regulator for the function of the RAS.

2. Methods

2.1. Animals

Age-matched male SHR and corresponding normotensive Wistar-Kyoto rats (WKY) were purchased (SLC Inc., Shizuoka, Japan). They had an adaptation period for one week in a constant room temperature (~ 21 °C) and humidity (~ 45 %) and a 12 h-12 h day and night cycle. Body weights of animals were 224.9 ± 4.9, 241.8 ± 3.8, and 243.4 ± 3.3 g for WKY-V group (n = 10), SHR-V group (n = 13), and SHR-ORS group (n = 12) at 7th week of age, respectively. Food (Cargillagripurina, Kunsan, Korea) and water were supplied ad libitum. Rats were housed individually in metabolic cages (Tecniplast, Buguggiate, Italy). All animal procedures for cares and experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Wonkwang University (WKU20–26) and all animal testing procedures were complied with the NIH *Guide for the Care and Use of Laboratory Animals*.

2.2. Experimental protocols

Experiments were performed using three groups of animals, normotensive WKY group treated with vehicle (WKY-V) and SHR groups treated with vehicle (SHR-V) or ORS water extract (ORS) (SHR-ORS). Animals received either vehicle (distilled water, 1 ml/day) or ORS (100 mg/kg/day dissolved in 1 ml of distilled water) orally (oral gavage) between 5:00 to 6:00 pm for 3 weeks, and were euthanized after 39 h on average of the last administration. Urine samples for night (6:00 p.m. through 9:00 a.m. next day) and day (9:00 a.m. through 5:00 p.m.) were collected separately. Changes in SBP were measured by tail-cuff plethysmography once a week during the experimental period in a quiet and comfortable room (MK-2000, Muromachi Kikai, Tokyo, JAPAN). Rats were killed with guillotine to avoid possible modulation by anesthesia[20,21] of the renal function or the RAS activity of the renal cortex. Blood was collected quickly in prechilled EDTA-coated or heparinized tubes. Plasma samples were separated and kept at −70 °C until used. Tissue samples for gene expression were snap frozen in liquid nitrogen and stored at −70 °C until used.

2.3. Preparation of ORS water extract

ORS water extract was prepared as previously reported.[3] Five medicinal herbs (*Alisma orientale* Juzepzuk, 9.375 g; *Poria cocos* Wolf, 5.625 g; *Atractylodes macrocephala* Koidzumi, 5.625 g; *Polyporus umbellatus* Fries, 5.625 g; *Cinnamomum cassia* Presl, 1.875 g; total weight to be 28.1 g of mixture of the dried herbs of ORS, a single dose for a human adult) were mixed. Mixture of the dried herbs of ORS (3.0 kg) was extracted in distilled water at 100 °C for 2 h using an electric extractor. The extract solution was filtered using a standard sieve, evaporated to dryness at 40 °C under vacuum, and freeze-dried. The amount of water extract was 681.2 g (yield, 22.7 %). Rats were administered with ORS water extract 100 mg/kg/day orally equivalent to a single dose for an average adult dose.[4]

2.4. Quantitative real time PCR

RT PCR analysis was performed as previously reported.[6,22] Total RNA of the cortex of the right kidney was extracted using TRIzol reagent (Ambion, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was synthesized using the High Capacity RNA-to-cDNA kit (Applied Biosystems, Waltham, MA, USA). For the RT PCR, initial denaturation step at 95 °C for 10 min was followed by 40 cycles at 95 °C for 15 s and finally at 60 °C for 60 s in the Step-One Real-Time PCR system (4,376,600, Applied Biosystems, Foster City, CA, USA). The RNA samples were measured in triplicate and normalized to the endogenous GAPDH. Data normalization of the gene expression was conducted by StepOne™ software version 2.3 (Applied Biosystems, Waltham, MA, USA). The specific sense and antisense primers used were as follows:[6,22] AT_1 receptor: S(5′-CTC AAG CCT GTC TAC GAA AAT GAG-3′), A(5′-TAG ATC CTG AGG CAG GGT GAA T-3′); AT_2 receptor: S(5′-GAA TCC CTG GCA AGC ATC TTA T-3′), A(5′-ATG TTG GCA ATG AGG ATA GAC AAG-3′); Mas receptor: S(5′-GGA TGC CAG AAT TGA ACA CAG A-3′), A(5′-CAC TGG CCC TCC TGA A-3′); ACE: S(5′-GGG CAT TGA CCT AGA GAC TGA TG-3′), A(5′-CTT GGG CTG TCC GGT CAT AC-3′); ACE2: S(5′-ACC AAA GCA TTA AAG TGA GGA TAA G-3′), A(5′- GTT GGT CCA TTC ATA TGC ATT-3′); NHE3: S(5′-CTG AGG AAC CGA GCA-3′), A(5′-AGG CCC AGA ACG ATG AGT AG-3′); ANP: S(5′- GAG AAG ATG CCG GTA G-3′), A(5′-CTA GAG AGG GAG CTA AGT G-3′); NPR-C: S(5′-TTC TGG CTT TGC ATG AAG TG-3′), A(5′-ATT TTG AAC CGG CCT TCT TT-3′); GAPDH: S(5′-GTC GGT GTG AAC GGA TTT G-3′), A(5′-CTT GCC GTG GGT AGA GTC AT-3′).

2.5. Chemical assay

Urinalysis was performed as previously reported.[22] Urine samples were centrifuged at 2000 xg for 15 min (4 °C) and supernatants were kept in a refrigerator. All chemical assays were completed within 12 h of sample collection. The concentration of ions was measured using

Electrolyte Analyzer (NOVA Biochemical, Waltham, MA, USA). Na^+ balance = Na^+ intake – urinary excretion of Na^+ ($U_{Na}V$). Na^+ intake = food consumed (g/23 h) x Na^+ content in food (μEq/g of food consumed). $U_{Na}V$ (μEq/23 h) = urine volume (ml/15 h) x Na^+ concentration in nighttime urine (μEq/ml) + urine volume (ml/8 h) x Na^+ concentration in day-time urine (μEq/ml). Creatinine concentration of plasma and urine was measured by colorimetric method (Jaffe reaction) using spectrophotometer (Miloton Roy, Rochester, NY, USA).

2.6. Preparation of aorta rings and measurement of vascular reactivity

Changes in vascular reactivity is one of the causes in the rise and maintenance of high blood pressure. Another series of experiments were conducted to determine changes in vascular reactivity by ORS. Aorta rings were prepared as reported previously.[23] Rats were killed by guillotine, exsanguinated, and thoracic aortas were dissected free from surrounding connective tissues and perivascular fats. For isometric wire myography, aorta was sliced into aorta rings (2.5 – 3 mm in length). Aorta rings were suspended in an organ chamber (5 ml, constant temperature at 37 °C) with oxygenated buffer solution at 1.2 g basal tension. The isometric forces of the rings were measured using a Grass force displacement transducer FT 03 connected to ADInstruments Power Lab 8SP physiology recording system (ADInstruments, Dunedin, New Zealand). The preparations were allowed to equilibrate for about 1 hour with replacement of buffer solution every 10 min. Vasodilation in response to cumulative doses of acetylcholine (ACh, 0.3 nM ~ 3 μM) was quantified in the phenylephrine (1 μM)-contracted aorta rings.

2.7. Statistical analyses

All results were presented as mean ± SEM. Statistical significance between groups were defined as $P < 0.05$. The significant differences between groups were validated by two-way ANOVA or repeated measures of ANOVA with Bonferroni's multiple comparison test or paired *t*-test. All statistical analyses were conducted using SigmaPlot 10.0 (SPSS Inc., Chicago, IL, USA), or GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Effects of ORS on the regulation of fluid and salt metabolism in SHR

Fig. 1 depicts daily changes in UV and $U_{Na}V$, and water and Na^+ balance in response to the treatment with ORS in normotensive WKY-V and hypertensive SHR-V or SHR-ORS. UV was significantly lower in the SHR-V group than the WKY-V treated with vehicle. During 3 control days before starting the administration of ORS, UV was lower in the SHR-V group than WKY-V (38.5 ± 1.0 vs. 80.3 ± 4.1 ml/kg/day; $n = 3$ control days; $P < 0.001$; Fig. 1A). UV of the SHR-ORS group was not significantly different from that of SHR-V. The levels of UV were stably maintained in the SHR-V group during the experimental period for 3 weeks and significantly lower than those of WKY-V ($P < 0.001$; Fig. 1A, C, and 2Aa). Chronic treatment with ORS (100 mg/kg, daily) significantly increased UV in the SHR-ORS group compared to SHR-V ($P \leq 0.001$; Fig. 1A, 1C, and 2Aa). The levels of water balance were not significantly different among the SHR and WKY-V groups during the 3 control days before ORS administration (Fig. 1B). The levels of water balance were not significantly different between the SHR-V group and WKY-V during the 2nd and 3rd weeks of the experimental period (Fig. 1B and 1C). Treatment with ORS significantly contracted the levels of water balance in the SHR-ORS group compared to SHR-V ($P < 0.01$; Fig. 1B and C). Fig. 1C shows a reciprocal relation between the changes in the urine volume and water balance in the SHR-V and SHR-ORS groups. That is, ORS induced an increase in UV leading to a decrease in water balance in the SHR-ORS group (Fig. 1A–C). The levels of $U_{Na}V$ were not significantly different among the three experimental groups during the 3 control days (Fig. 1D). The levels of $U_{Na}V$ were not significantly different between SHR-V and WKY-V during the first week but the levels of SHR-V group were significantly lower than those of WKY-V during the 2nd and 3rd week of the experiment (2nd week, 5.95 ± 0.15 vs. 6.65 ± 0.15; 3rd week, 5.68 ± 0.20 vs. 6.56 ± 0.14 mEq/kg/day, $n = 7$ days for both groups, $P < 0.01$ vs WKY-V; Fig. 1D and F; Fig. 2Ab). Treatment with ORS significantly increased $U_{Na}V$ in the SHR-ORS group compared to SHR-V (3rd week, 8.16 ± 0.23 vs. 5.68 ± 0.20 mEq/kg/day, $n = 7$ days for both groups, $P < 0.001$; Fig. 1D and F; Fig. 2Ab). The levels of Na^+ balance were not significantly different among the groups during the 3 control days (Fig. 1E). The levels of Na^+ balance were significantly higher in the SHR-V group than the WKY-V during the 3rd week of the experimental period (5.31 ± 0.19 vs. 3.64 ± 0.18 mEq/kg/day, $n = 7$ days for both groups, $P < 0.001$; Fig. 1E and F). Treatment with ORS contracted the levels of Na^+ balance in the SHR-ORS group compared to SHR-V (2.98 ± 0.25 vs. 5.31 ± 0.19 mEq/kg/day, $n = 7$ days for both groups, $P < 0.001$; Fig. 1E and 1F). Fig. 1F shows a reciprocal relation between $U_{Na}V$ and Na^+ balance in the SHR-V and SHR-ORS groups. That is, an increase in $U_{Na}V$ leads to a decrease in Na^+ balance in the SHR-ORS group. These findings show that the treatment with ORS increased UV and $U_{Na}V$ leading to a contraction of the water and Na^+ retention. Fig. 2 shows summarized effects of ORS on the renal excretory function and GFR during the 3rd week of the treatment with ORS in the SHR. The levels of UV, $U_{Na}V$, and clearance for Na^+ (C_{Na}) were lower in the SHR-V group than WKY-V (Fig. 2Aa, 2Ab, and 2Bb). ORS increased UV, $U_{Na}V$, C_{Na}, U_KV, and $U_{Cl}V$ in SHR-ORS group (Fig. 2A and B). These findings were accompanied by an increase in C_{Cr} (GFR) but not FE_{Na}% changes (Fig. 2B). The levels of $U_{Na}V$ and C_{Na} were lower in the SHR-V group compared to those of WKY-V. Treatment with ORS increased the levels of $U_{Na}V$ and clearance for Na^+ (C_{Na}) concomitantly with an increase in GFR. However, the levels of FE_{Na}% changes were not significantly increased in the SHR-ORS group (Fig. 2B).

3.2. Effects of ORS on impaired vasodilation in SHR

Fig. 3A shows effects of ORS on vasodilation in aorta from SHR. Fig. 3A shows an impaired response of aorta strips to acetylcholine (ACh). ACh induced vasodilation in a concentration dependent manner in aorta from WKY-V. However, the ACh-induced vasodilation was impaired in aorta from SHR-V compared to the aorta from WKY-V. Chronic treatment with ORS ameliorated the impaired ACh-induced vasodilation in SHR-ORS group. As shown in Fig. 3B, impaired vasodilation observed in the SHR-V group was associated with an accentuation of abundance of AT_1 receptor and suppression of AT_2 receptor expression in the aorta. Treatment with ORS restored the ACh-induced vasodilation with suppression of AT_1 receptor gene expression and accentuation of AT_2 receptor expression in the SHR-ORS group.

3.3. Effects of ORS on systolic blood pressure

Fig. 4 shows effects of chronic treatment with ORS on the regulation of SBP in SHR. Basal levels of SBP observed in the SHR-V group treated with vehicle was significantly higher than those of the WKY-V group at the age of 7th week and further increased as a time-dependent function (200.3 ± 3.6 vs. 120.3 ± 1.2 mmHg, $P < 0.001$). Increase in SBP significantly different from that in WKY-V. Treatment with ORS decreased SBP in the SHR-ORS group compared to the SHR-V group (126.9 ± 5.1 vs. 200.3 ± 3.6 mmHg; $P < 0.001$ at the 11th week of after birth). The effect was significant after one week of ORS administration. Up to three weeks of the treatment with ORS gradually further decreased SBP in the SHR-ORS group. SBP in the WKY-V group increased slightly but significantly as a time function (120.3 ± 1.2 vs. 108.6 ± 2.5 mmHg; $P < 0.05$ at the 11th week).

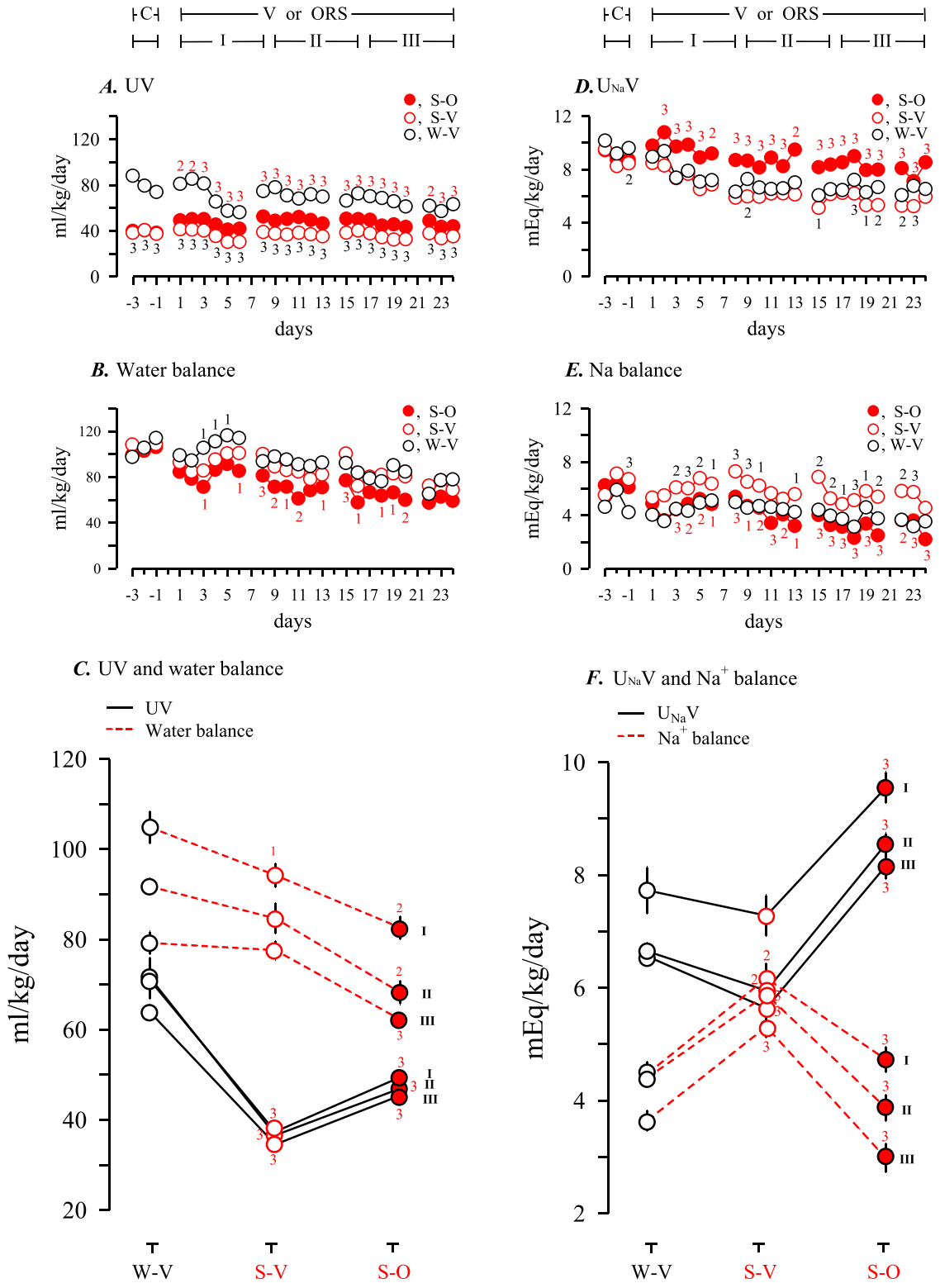

Fig. 1. Effects of chronic treatment with ORS on the daily changes in urine volume (UV, *A*) and water balance (*B*), and urinary excretion of Na^+ ($U_{Na}V$, *D*), and Na^+ balance (*E*), in the normotensive Wistar Kyoto rats (WKY-V) and spontaneously hypertensive rats (SHR-V or SHR-O), respectively, treated with vehicle (V) or ORS (O). Summarized changes in the UV and water balance (*C*) and $U_{Na}V$ and Na^+ balance (*F*) are also shown. Values are the means ± SEM of 7 days of 1st (I), 2nd (II), and 3rd week (III) of the experiments. Days −3 ~ −1, control days (C) before administration of ORS are followed by phases (I, II, and III) of vehicle or ORS treatment. Vehicle or ORS (1 ml) was administered orally during the experimental period (days 1 ~ 24). The dose of ORS was 100 mg/kg/day which is an equivalent amount of a single dose of ORS for an adult subject [4]. Numerals above or below the markings; 1, $P < 0.05$; 2, $P < 0.01$; 3, $P < 0.001$ vs corresponding controls, WKY-V or SHR-V. Numerals in red are significance test between the SHR-ORS and SHR-V groups and those in black are the tests between the SHR-V and WKY-V. Number of experiments for the comparisons between the groups in (A), (B), (D) and (E); WKY-V, $n = 10$; SHR-V, $n = 13$; SHR-O, $n = 12$. Number of experiments for the comparison between groups in (C) and (F); $n = 7$ for mean ± SEM of 7 days of 1st (I), 2nd (II), and 3rd week (III) of vehicle or ORS treatment.

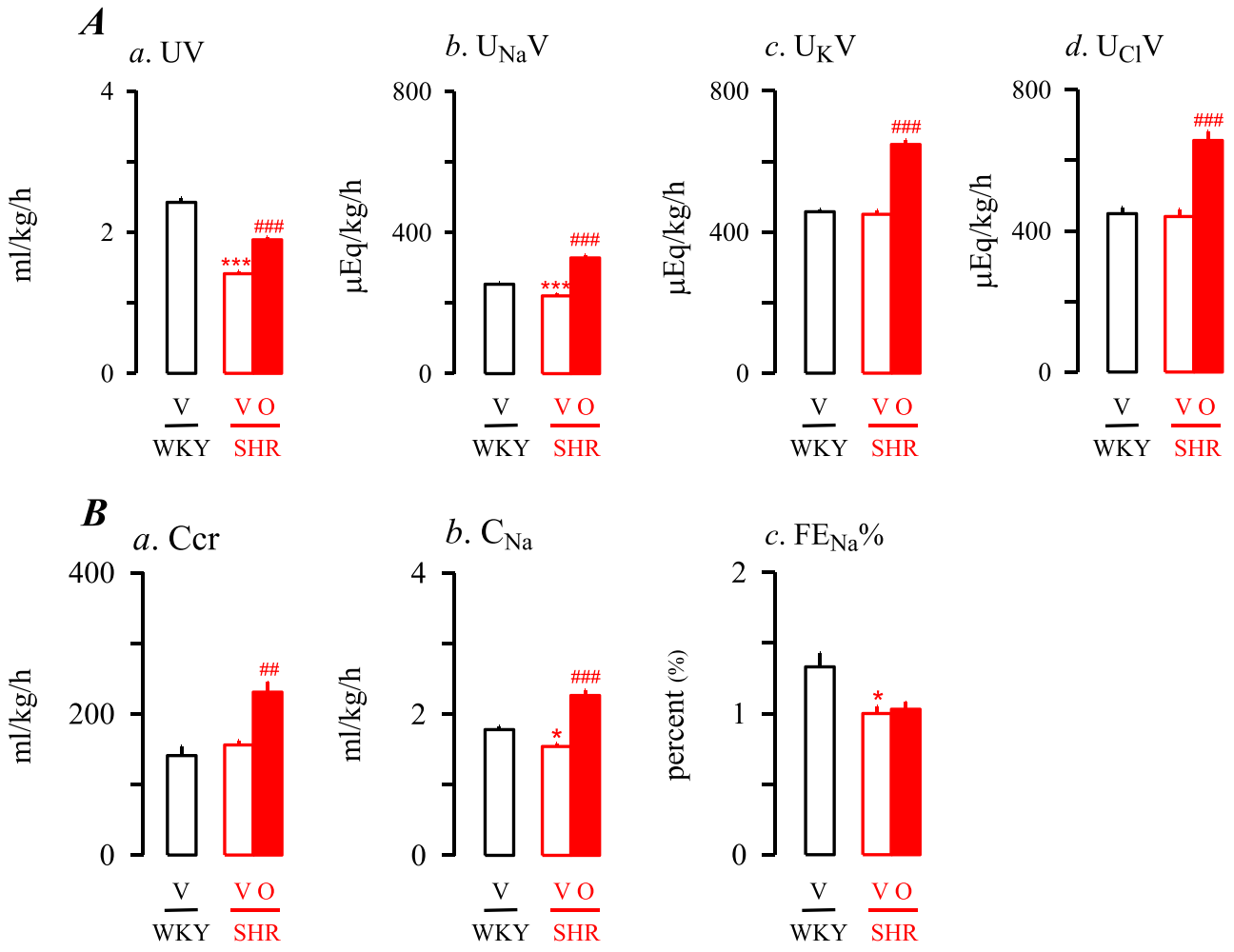

Fig. 2. Summarized effects of chronic treatment with ORS on the (*A*) urinary volume (UV, *a*), urinary excretion of Na^+ ($U_{Na}V$, *b*), K^+ (U_KV, *c*), and Cl^- ($U_{Cl}V$, *d*) and (*B*) clearance for creatinine (C_{Cr}, glomerular filtration rate, GFR, *a*), clearance for Na^+ (C_{Na}, *b*), and percent changes in the fractional excretion of Na^+ (FE_{Na}%, percent changes in C_{Na}/GFR, *c*) during the last 7 (*A*) or 3 (*B*) days (of the third week) of ORS treatment. V, vehicle-treated; O, ORS-treated. $^*P < 0.05$, $^{***}P < 0.001$ vs. WKY-V.; $\#\#P < 0.01$, $\#\#\#P < 0.001$ vs. SHR-V; Number of experiments: $n = 7$ days (last 3rd week) for WKY-V, SHR-V, and SHR-ORS groups for 2*A; n* = 3 days (last 3 days) for each group of 2*B* For the calculation of the levels of the GFR and C_{Na} of the last 3 days, the last day plasma samples were used.

3.4. Effects of ORS on intrarenal expression of components of the RAS, NPS, and NHE3

Fig. 5 shows effects of ORS on the expression of components of the RAS and NPS, and NHE3 in the renal cortex from SHR and WKY. Abundance of the AT_1 receptor gene expression was increased in the renal cortex from SHR-V compared to that of WKY-V (Fig. 5A). Abundance of the ACE gene expression tended to increase in the renal cortex from SHR-V. Treatment with ORS decreased the ACE and AT_1 receptor gene expression from SHR-ORS group. In contrast, AT_2 receptor, ACE2, and Mas receptor gene expressions were decreased in the renal cortex from the SHR-V group and chronic treatment with ORS increased abundance of these gene expressions in the SHR-ORS group (Fig. 5B). Na^+-coupled ion transporter NHE3 gene expression was increased in the renal cortex of the SHR-V group compared to that of WKY-V and the treatment with ORS decreased the expression in the SHR-ORS group (Fig. 5C). ANP gene expression was decreased in the renal cortex of the SHR-V group, while natriuretic peptide receptor (NPR)-C expression tended to increase (Fig. 5C). Chronic treatment with ORS reversed the expression leading to an increase in abundance of the ANP gene expression and a decrease in NPR-C gene expression in the renal cortex from SHR-ORS group.

4. Discussion

The present study shows that ORS accentuated the renal roles for the Na^+ and water balance and decreased SBP in SHR-ORS group. The site of action for ORS was mainly in the renal cortex where the proximal tubule is located. The results show that the Na^+ balance in the body is maintained at higher levels in the SHR-V group compared to the control WKY-V in this experimental condition. Treatment with ORS contracted the levels of Na^+ balance in SHR-ORS group and reversed the values toward those of the normotensive WKY-V. Contraction of the Na^+ balance by ORS in SHR-ORS group was closely associated with an increase in urinary excretion of Na^+ ($U_{Na}V$) (Fig. 1, D and F). These findings show that ORS contracts Na^+ balance with an increase in urinary excretion of Na^+ and decrease in abundance of NHE3 expression along with suppression of the ACE-AT_1 receptor signaling pathway and concurrent accentuation of AT_2 receptor/ACE2-Mas receptor pathways (Fig. 6). These findings are consistent with the previous reports. Previously, it was shown that AT_2 receptor activation with non-peptide agonist Compound-21 resulted in an increase in AT_2 receptor at the apical membrane of the renal proximal tubule cells and internalized/inactivated NHE3 in Sprague-Dawley rats.[16,24] Recently, it was further shown that activation of AT_2 receptor with an inhibition of NHE3 signaling increased urinary excre-

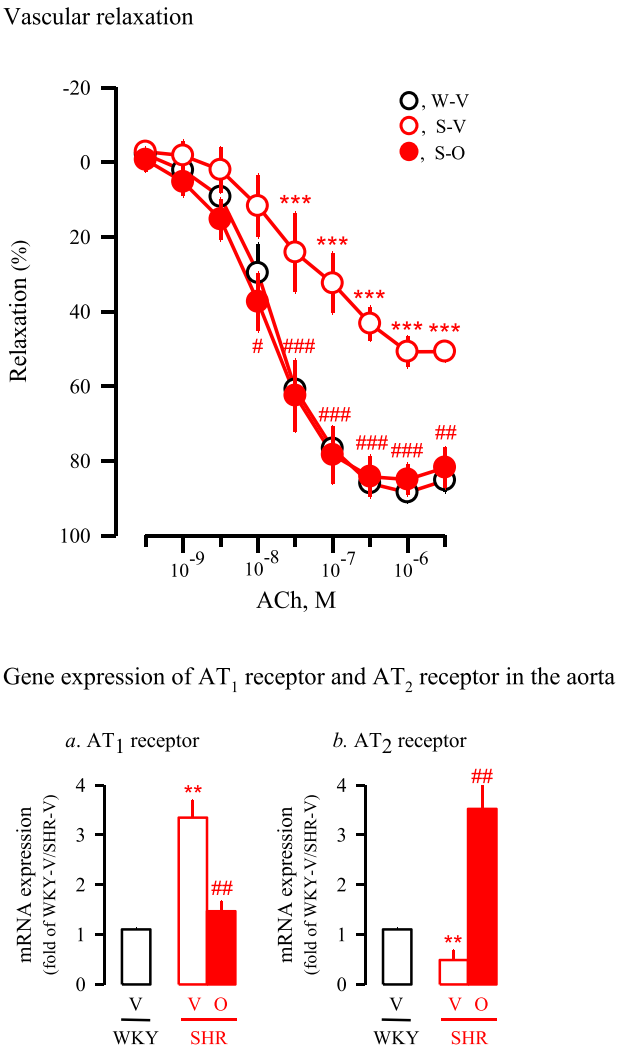

Fig. 3. Effects of chronic treatment with ORS on the impaired vasodilation (*A*) and gene expression of components of the renin-angiotensin system (*B*) of the aorta from SHR. *A*. Acetylcholine induced vasodilation was significantly suppressed and shifted right-upward in the aorta from SHR-V group (S-V) compared to that of normotensive WKY-V group (W-V). Chronic treatment with ORS reversed the suppression and shifted the response toward left-downward in the SHR-ORS group (S-O) compared to the SHR-V group. Number of experiments; WKY-V, $n = 4$, SHR-V, $n = 4$, SHR-ORS, $n = 4$. $^{***}P < 0.001$ vs. WKY-v; $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$ vs. SHR-V. *B*. Impaired vasodilation of the aorta was accompanied by combined increase in the AT_1R gene expression and decrease in the AT_2R expression in the aorta from SHR-V. Chronic treatment with ORS reversed abundance of the gene expression in the aorta from SHR-ORS. $^{**}P < 0.01$ vs. WKY-V; $\#\#P < 0.01$ vs. SHR-V.

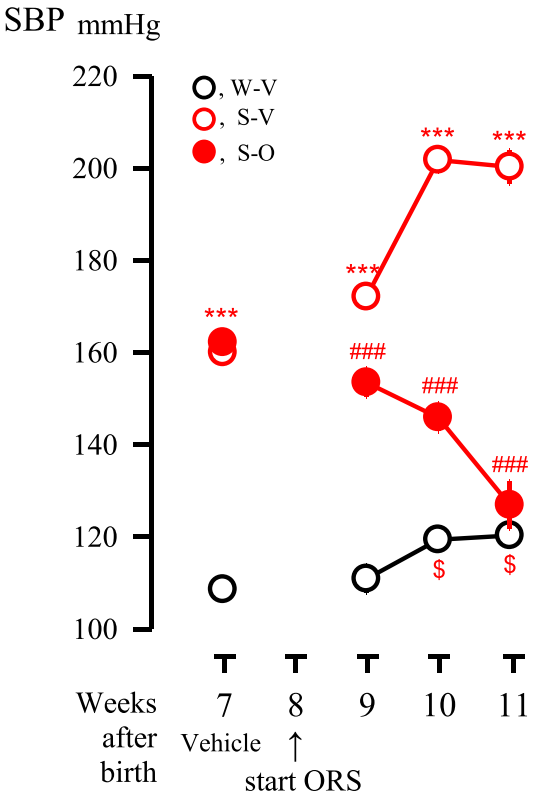

Fig. 4. SBP significantly increased as a time function in SHR-V group compared to WKY-V. Chronic treatment with ORS (100 mg/kg/day, orally) significantly decreased SBP in SHR-ORS group compared to SHR-V. After 1 week of treatment with ORS the decrease was significant and further decreased up to 3rd week of the treatment in the SHR-ORS group. SBP of the normotensive WKY-V group slightly but significantly increased. Number of experiments; WKY-V, $n = 4$: SHR-V, $n = 6$: SHR-ORS, $n = 5$. $^{***}P < 0.001$ vs. WKY-V; $\#\#\#P < 0.001$ vs. SHR-V. $\$P < 0.05$ vs. 7th week. Weeks 7-11 are weeks after birth of rats used.

tion of Na^+via action of protein phosphatase 2A (PP2A) in the renal proximal tubule cells in WKY but not in SHR.[14]

AT_2 receptor-NHE3 signaling is known to be defective in the proximal tubule cells of the kidney from pre-hypertensive/hypertensive SHR.[12–14] That is, natriuresis induced by AT_2 receptor activation (with renal interstitial infusion of Ang III[12] or Compound-21[13,14]) is impaired in SHR. This suggests that, AT_2 receptor-NHE3 signaling pathway for the natriuresis is defective in the SHR which may be associated with hypertension in this model. In the present study, UV and urinary excretion of Na^+ were suppressed in the SHR-V group and Na^+ balance was maintained at the higher levels compared to control WKY-V. These were accompanied by an increase in abundance of ACE-AT_1 receptor and NHE3 expression and a suppression of AT_2 receptor/ACE2-Mas receptor expression in the renal cortex of SHR-V group (Fig. 6). Activation of Ang II-AT_1 receptor signaling pathway in the proximal tubule is an important factor leading to an accumulation of Na^+ and water[25–29] and activates NHE3 located at this segment.[29] Treatment with ORS reversed all these findings to induce natriuresis, diuresis and contraction of the Na^+ and water balance, and decrease of SBP in the SHR-ORS group. The RAS and NHE3 located at the renal proximal tubule are associated with the regulation of Na^+ reabsorption and non-redundant role for the blood pressure homeostasis.[16,19,30] Changes in the urinary Na^+ and water excretion, therefore, Na^+ and water balance in body, is closely associated with the regulation of the blood pressure homeostasis.[9–11] The present study showing an antihypertensive effect of ORS through modulation of the RAS-NHE3 signaling in the renal cortex supports the previous observations in the Goldblatt hypertensive rats.[6] Activation of ACE-AT_1 receptor-NHE3 signaling pathway induces anti-natriuresis, Na^+ accumulation, and hypertension. In contrast, activation of the Ang III-AT_2 receptor pathway is antagonistic to the ACE-AT_1 receptor activation in general and AT_2 receptor-NHE3 signaling induces natriuresis and lowering of SBP.[15–19] The proximal tubule contains all of the components of the RAS.[31,32] Altogether, ORS decreased body water and Na^+ retention leading to lowering of high blood pressure through suppression of abundance of NHE3 and AT_1 receptor gene expression and accentuation of AT_2 receptor expression in the renal cortex (proximal tubule). These notion supports the difference between ORS (present study) and Compound-21[14] in the control of the RAS signaling pathways, AT_2R-NHE3 signaling pathway in the renal proximal tubules from SHR in particular. Significance of the present study is that the treatment with ORS ameliorates hypertension through broad action mechanisms: modula-

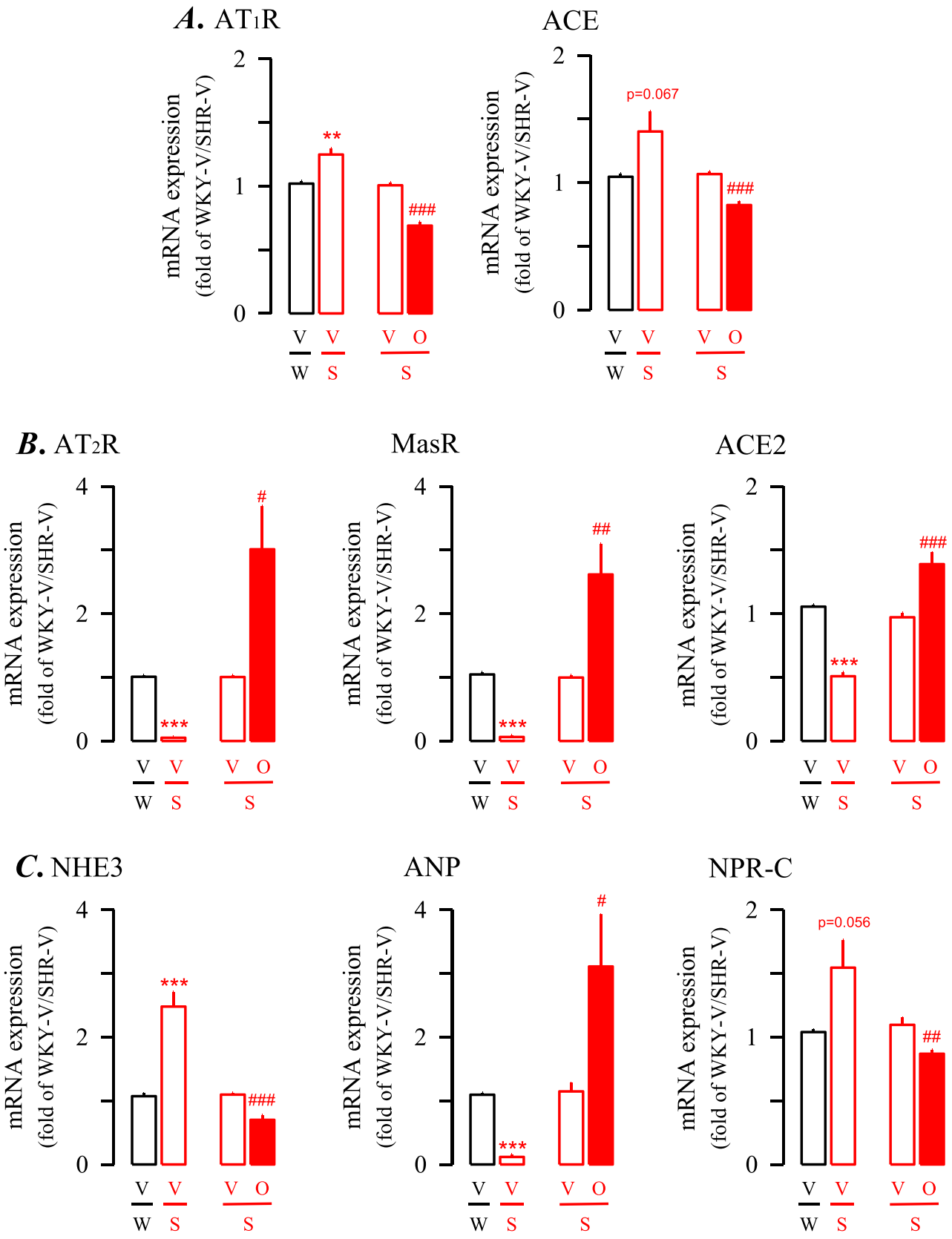

Fig. 5. Effects of chronic treatment with ORS on the gene expression of the components of the RAS, NPS and NHE3 in the cortex of the right kidney from SHR and WKY. Assay was performed in triplicate. Number of experiments; $n = 6$ for W (WKY)-V, $n = 7$ for both S (SHR)-V and S (SHR)-O (ORS). There are two open red bars in succession with different meanings in S-V group. First one is normalized compared to the values of the normotensive W-V group, and the other is normalized compared to hypertensive group treated with vehicle. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. W-V; $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$ vs. SHR-V.

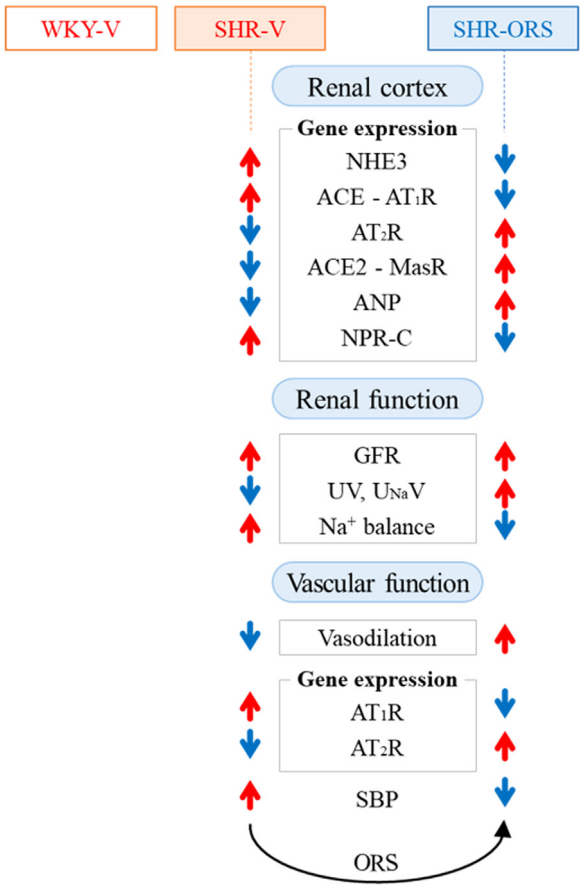

Fig. 6. Antihypertensive effects of ORS through modulation of the renal cortical function and vasodilation via the RAS, NPS and NHE3 signaling in SHR. Chronic treatment with ORS decreased Na^+ and water retention through inhibition of the NHE3 expression along with suppression of the ACE-AT_1 receptor expression and accentuation of the AT_2 receptor, Mas receptor, and modulation of the NPS in the renal cortex (proximal tubule) of the SHR. In addition, ORS treatment ameliorated defective vasodilation through modulation of the AT_1 receptor and AT_2 receptor in the vascular bed. These findings provide experimental evidence showing that chronic treatment with ORS decreases high blood pressure through improving body fluid volume and pressure homeostasis and partly vasodilation in the SHR. NHE3, Na^+-H^+ exchanger 3; AT_1R, angiotensin II type 1 receptor; AT_2R, angiotensin II type 2 receptor; ANP, atrial natriuretic peptide; NPR-C, natriuretic peptide receptor C; GFR, glomerular filtration rate; $U_{Na}V$, urinary excretion of Na^+; SBP, systolic blood pressure; ORS, Oryeongsan.

tion of renal excretory function (present study), atrial ANP secretion,[3] and vascular responsibility (present study) in the SHR. Further, it is an important finding that ORS suppresses expression of the AT1R-NHE3 signaling pathway in the kidney, because NHE3 expression is upregulated in the proximal tubule or the medulla of the kidney.[33] However, the mechanisms involved in the modulation by ORS of the expression and action for the AT_1 receptor and/or AT_2 receptor in the renal cortex are remains to be defined.

Although the etiology of the rise of hypertension is different between Goldblatt hypertensive rats, an animal model of human secondary hypertension, and SHR hypertensive rats, an animal model of human essential hypertension, the effects of ORS are similar in both experimental models. We studied the effects of ORS on the body fluid and Na^+ metabolism in association with the RAS and NPS in the Goldblatt hypertensive rats[6] and SHR (present study). The reason of the similar effects of ORS in both models of hypertension may be that the animal models are renin-dependent hypertensive models[6,34,35] and major mechanism of action of ORS are modulation of the renin-angiotensin system. Maintenance of hypertension is associated with an accentuation of AT1R and NHE3 expression in the kidneys from both Goldblatt hypertensive rats[6] and SHR (present study) and ORS ameliorates high blood pressure via reversion of the changes in the RAS with NHE3 expression in the renal cortex.

The regulation of NHE3 signaling is also closely associated with the action of the NPS in the renal proximal tubule. As shown in the present study, ORS accentuated ANP gene expression and suppressed ANP clearing receptor NPR-C expression in the cortex of the kidney from SHR-ORS group which may lead to an increase in the local ANP concentration in the proximal tubule. Because ANP reduces the proximal tubule reabsorption through inhibition of the NHE3 located at the luminal membranes of the renal proximal tubule,[36,37] it is possible that ORS modulates the Na^+ reabsorption via the NPS-NHE3 signaling in the renal proximal tubule. In the proximal convoluted tubule from human kidney, major components of the NPS including pro-ANP processing corin, pro-ANP/ANP, selective ANP receptor NPR-A, and ANP degrading protease neprilysin are expressed.[35] These findings suggest that the presence of the ANP autocrine signaling system has a role for the regulation of the Na^+ and water reabsorption and blood pressure homeostasis. Therefore, it is possible that ORS affects the regulation of volume and pressure homeostasis via ANP autocrine signaling system in this renal segment. Peritubular perfusion of ANP inhibits the Ang II-stimulated Na^+ and water reabsorption in the proximal tubule from rats.[38,39]

As for the increase in GFR by ORS, it was shown that acute, subacute, or chronic treatment with ORS increased GFR in experimental animals.[1,2,6] As shown in the present study, chronic treatment with ORS increased GFR concomitantly with an increase in abundance of the ANP gene expression and suppression of the ANP clearing NPR-C receptor in the cortex of the kidney. Locally generated ANP could be involved in the regulation of the proximal tubule ANP levels. ANP is known to increase whole kidney and single nephron GFR through afferent arteriolar vasodilation and concurrent efferent arteriolar vasoconstriction in the anesthetized rats.[40] Further, it was also shown by measuring the changes in the diameter of the microvessels that ANP induces preglomerular vasodilation and efferent arteriolar constriction (leading to an increase in GFR) in hydronephrotic kidney of rats.[41] Also, ANP induces afferent preglomerular vasodilation and efferent arteriolar vasoconstriction in the isolated afferent and efferent arterioles from rats.[42] ORS-induced accentuation of atrial secretion of ANP is also expected to be involved.[3]

The present study shows an impaired vascular function in SHR. ACh-induced vasodilation was impaired in the aorta strips from the SHR-V group in which AT_1 receptor expression was accentuated and AT_2 receptor was suppressed compared to control WKY-V. Chronic treatment with ORS ameliorated the failure of the vasodilation with suppression of AT_1 receptor gene expression and accentuation of AT_2 receptor expression. Previously, it was shown that impaired ACh-induced vasodilation was ameliorated by the treatment with AT_1 receptor blockade losartan in mouse carotid artery.[43]

The mechanisms involved in the decrease in SBP of SHR by treatment with ORS could be three-fold: one is correction by ORS of the impaired Na^+ and water balance, and the other is amelioration of the impaired vasodilation and then accentuation of ANP secretion from the atria.[3] Chronic treatment with ORS decreased NHE3 expression concomitantly with an accentuation of AT_2 receptor and suppression of AT_1 receptor in the renal cortex leading to a decrease in Na^+ and water retention in the SHR-ORS group. Regulation of the basal blood pressure and the development of the high blood pressure are closely associated with the regulation of the urinary flow and excretion of Na^+ and body Na^+ and water balance through the renal function.[9]

Chronic treatment with ORS increased $U_{Na}V$ along with GFR, but the FE_{Na}% changes were not increased. This suggests that the role of an increase in GFR may not be excluded as one of the mechanisms, at least in part, for the ORS induced increase in urinary excretion of Na^+in the SHR-ORS group.

In conclusion, urinary volume and excretion of Na^+ were suppressed in the SHR-V group compared to normotensive WKY-V. The levels of Na^+ retention were higher in the SHR-V than WKY-V. Chronic treatment with ORS contracted the Na^+ and water retention along with an increase in UV and $U_{Na}V$ in the SHR-ORS group compared to SHR-V and decreased SBP significantly. These findings were associated with a decrease in abundance of NHE3 expression along with suppression of ACE-AT_1 receptor and concurrent accentuation of AT_2 receptor/ACE2-Mas receptor expression in the renal cortex of the SHR-ORS group. These findings provide pharmacological evidence for the ORS effects in amelioration of hypertension through promotion of the Na^+ balance via modulation of the proximal tubule AT_1 receptor-NHE3 pathway and ACE2-Mas receptor/AT_2 receptor-NHE3 signaling pathway in the renal cortex of SHR.

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CRediT authorship contribution statement

You Mee Ahn: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Hye Yoom Kim:** Methodology, Software, Validation, Formal analysis, Investigation, Writing – original draft, Data curation, Visualization, Funding acquisition. **Dae Gill Kang:** Conceptualization, Writing – review & editing, Project administration. **Kyung Woo Cho:** Conceptualization, Data curation, Writing – original draft, Writing – review & editing, Visualization, Project administration. **Ho Sub Lee:** Conceptualization, Writing – review & editing, Project administration, Funding acquisition.

Conflict of interest

All authors declare that there are no conflicts of interest.

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Ethical statement

Not applicable.

Data availability

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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