**Activation of the intestinal tissue renin-angiotensin system**

**by transient sodium loading in salt-sensitive rats.**

Data supplement

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**Extended Materials and Methods**

***Experiments***

All the experiments were approved by the institutional review committee and performed in accordance with the Keio University School of Medicine Animal Experimentation Guidelines, based on the Declaration of Helsinki.

Male spontaneously hypertensive rats (SHR-izm), Wistar Kyoto rats (WKY-izm), and Dahl salt-sensitive rats (DIS/Eis Slc) were purchased from Sankyo Laboratory Services (Tokyo, Japan) and maintained in a temperature-controlled room at 23°C with 12:12-hour light-dark cycles under specific pathogen-free conditions. All the rats were allowed free access to standard rat chow (CE-2; CLEA Japan, Inc., Tokyo, Japan) containing sodium (0.26 g/100 g) and potassium (1.06 g/100 g).

Spontaneously hypertensive rats (SHRs) are known to exhibit hypertension with many features that are also observed in essential hypertension in human beings, such as increased cardiac output, elevated sympathetic nervous activity, and cardiovascular hypertrophy[1-3]. According to our previous study, although both SHRs and Dahl salt-sensitive rats (DS rats) showed blood pressure (BP) elevation after salt loading, both the models could be termed salt-sensitive hypertension models; increase in the basal BP without salt loading was only observed in SHRs, but not in DS rats[4]. In salt-resistant Wistar Kyoto (WKY) rats, BP showed no significant response to salt loading[5]. Additionally, the DS rats are known to be prone to hypertensive nephropathy, and do not reflect the relatively slow development of renal damage in human hypertension[6,7]. Accordingly, SHRs rather than DS rats seemed to be a useful model for human hypertension; therefore, we selected SHRs for Experiment 1.

Transient salt loading on weanling Dahl salt-sensitive rats for 6 weeks induced permanent hypertension[8]. In the present study, randomly selected rats were treated with high salt (1% NaCl) water from 6 to14 weeks of age, which can be considered pre-middle age when high BP is established, in line with our previous report[4]. During the medication period, the angiotensin II (Ang II) type 1 receptor blocker (ARB)—that is, valsartan (30 mg/kg pellet; MedKoo Biosciences Inc., Morrisville, NC)—was administered by mixing the drugs into the chow diets. By administrating this ARB dosage, we expected a decrease of 30–40 mmHg in the BP value; this can be considered high-dose pulse treatment, according to previous reports[9,10].

***Assays***

The systolic BP of the rats was measured by using a tail-cuff microsensor device (model MK-2000, Muromachi Kikai, Tokyo, Japan). The average of four measurements was determined for each rat. The total body water amount was measured using an impedance method with ImpediVET (ImpediMed Inc., Carlsbad, CA)[11]. The *in vivo* body composition of rats determined using ImpediVET shows a good correlation with a chemical carcass analysis for the total body water amount[11]. All the measurements were performed during a constant 3-hour period (from 1 pm to 4 pm). The rats were placed in metabolic cages for 24 hours to determine their food and water consumption and to collect their urine and fecal samples. Stool samples were processed to measure the fecal sodium content, as reported previously[12]. Briefly, the samples were weighed after drying them at 80°C for 3 hours, suspended them in 0.75 mol/L nitric acid, incubated them overnight at 4°C, and centrifuged them to obtain the supernatants that were used for the measurements.

Plasma renin activity (PRA), plasma aldosterone concentration (PAC), plasma Ang II, serum atrial natriuretic peptide (ANP), serum urea nitrogen, and serum creatinine, as well as urinary sodium and the supernatant of fecal sodium suspension were measured using a standard method (SRL Inc., Tokyo, Japan). Plasma Ang II was measured using an Ang II radioimmunoassay kit (Fujirebio Inc., Tokyo, Japan). Intestinal Ang II concentration was determined as reported previously[13]. In brief, the tissues were boiled for 10 minutes in distilled water (10 mL/g of tissue). After boiling, acetic acid was added to the samples to achieve a final concentration of 1.0 mol/L. Subsequently, the samples were homogenized and immediately centrifuged at 12,000 rpm for 20 minutes at 4°C. The supernatant of the samples was applied to a Sep-Pak C18 cartridge and eluted with 60% acetonitrile in 0.1% trifluoroacetic acid. The eluted samples were lyophilized and stored at –20°C until measurement. Tissue Ang II concentration was determined using an Ang II Elisa kit (EKE-002-12; Phoenix Pharmaceuticals Inc., Burlingame, CA).

***RNA extraction and real-time quantitative PCR analysis***

To explore the impact of salt loading and renin-angiotensin system (RAS) inhibition, gene expression of the intestinal and renal sodium transporters and RAS components was examined. Total RNA was extracted using a kit (74104, RNeasy Mini Kit; Qiagen, Hilden, Germany). Complementary DNA was synthesized via reverse transcription of RNA using a kit (RR037A, Primescript RT Reagent Kit; Takara Bio Inc., Otsu, Japan). Complementary DNA was used for quantitative real-time PCR (RT-qPCR) using fluorescent dye reagent (SYBR Premix Ex Taq ll; Takara Bio Inc.) and a real-time PCR system (7500 Fast; Applied Biosystems, Waltham, MA). The mRNA levels were measured and normalized to those of 18S ribosomal RNA. All the gene expressions are shown as relative expressions. The sequences of primers used in RT-qPCR are listed below.



***Analysis of intestinal and renal histological features***

The protein staining intensity of intestinal sodium transporters was examined in line with the gene expression. Histological features of the renal arteriole were examined as well. Intestinal histological features were evaluated at 14 and 28 weeks of age, and renal histological features were evaluated at 28 weeks of age. Immunohistochemical staining of small intestine samples for sodium/hydrogen exchanger isoform 3 (NHE3) and sodium glucose cotransporter 1 (SGLT1) was performed as follows. Formalin-fixed paraffin-embedded sections with 4 µm thickness were deparaffinized and hydrated, and antigens were retrieved (415211, Histofine Antigen Retrieval Solution pH9; Nichirei Biosciences, Tokyo, Japan). Endogenous peroxidase was blocked with 0.3% H2O2 in methanol for 30 minutes. To reduce nonspecific binding, sections were incubated with 2.5% normal horse serum for 30 minutes. After washing with PBS, the sections were incubated with anti-NHE3 rabbit polyclonal antibody (ab95299; Abcam, Cambridge, UK) diluted to 1/100 or anti-SGLT1 rabbit polyclonal antibody (ab14685; Abcam) diluted to 1/100 for 1 hour. After washing with PBS, sections were incubated for 30 minutes using an ImmPRESS HRP anti-rabbit IgG (MP-7401-15; Vector Laboratories, Burlingame, CA). The staining was visualized with 3,3’-diaminobenzidine (DAB) for 4 minutes. Sections from the rat kidneys were stained with Masson’s trichrome stain (HT15; Sigma-Aldrich, St. Louis, MO) for the assessment of medial hyperplasia of the renal arterioles.

***Quantitative analysis of intestinal and renal histological features***

To evaluate the histological features in the small intestine and kidney, all the images were scanned using Nanozoomer-XR (Hamamatsu Photonics K.K., Shizuoka, Japan). The degree of positive expression of NHE3 and SGLT1 was analyzed using Adobe Photoshop. For each section, 11 parts from 3 fields of the cilia of the intestinal epithelia were randomly selected. To evaluate the positive expression of NHE3 and SGLT1 immunostaining, the density in the positive epithelial cells was evaluated using Adobe Photoshop, as described previously[14]. In the renal slides, blood vessels (small-medium sized arterioles) were evaluated using 11 randomly selected fields per cross-section from the kidneys across all four groups. The arteries were measured and graded as described previously[4,15].

***Cell culture***

To investigate whether the activation of intestinal RAS leads to induction of genes encoding for sodium transporters in the gut, Caco-2, human colorectal adenocarcinoma derived epithelial cells, were treated with either Ang II, NaCl, or aldosterone. Caco-2 cells were acquired from RIKEN BioResource Research Center (Tsukuba, Japan). While Caco-2 cells are derived from colorectal adenocarcinoma, the cells phenotypically and functionally resemble small intestinal enterocytes[16]. Caco-2 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, no.11995; Gibco, Waltham, MA), which was supplemented with 10% fetal bovine serum (FBS). The Caco-2 cells were incubated with 0.1% FBS for 12 hours before the experiments. Then, the Caco-2 cells were treated with Ang II (10-10, 10-9, and 10-8 mol/L) with or without AT1R blocker, valsartan (Ang II, 10-8 mol/L; valsartan, 10-5 mol/L) or AT2R antagonist, PD123319 (Ang II, 10-8 mol/L; PD123319, 10-5 mol/L), NaCl (10-2, 5×10-2, and 10-1 mol/L), or aldosterone (10-10, 10-9, and 10-8 mol/L) for 24 hours. Mannitol was prepared so that it had the same osmotic pressure as NaCl.

The treatment concentration of Ang II was determined according to the Ang II levels in the plasma or intestine, while the treatment concentration of NaCl was set considering the Na+ and Cl- concentrations in the intestine, which are known to range from 60 to 140 mM[17]. The agents were obtained from Sigma-Aldrich (valsartan, PD123319, NaCl, and mannitol) and Peptide Institute, Suita, Japan (Ang II and aldosterone).

**Supplementary figure legends**

**Figure S1.** **Transient salt loading for 8 weeks causes a sustained decrease in the fecal sodium content.** (A) Time-dependent shift of sodium balance in spontaneously hypertensive rats. Quantity of sodium output (in urine and feces) at 14, 21, and 28 weeks of age is normalized as the ratio by the quantity of sodium intake at each age. Data were taken from the experiments summarized in Figure 1. For each rat group (tap, salt, ARB), the average sodium intake through food (gray box) is compared with the urinary sodium output (white box) and fecal sodium output (black box). Gray box: sodium intake; white box: urinary sodium output; black box: fecal sodium output; n = 16 per group.

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**Figure S2. Effects of salt loading for 8 weeks in Wistar Kyoto rats.** (A) Systolic BP. (B) Body weight. (C) Total body water examined using an impedance method. (D) Water and food consumption. (E) Urinary volume and stool weight. (F) Urinary and fecal sodium excretion. (G) Plasma renin activity (PRA), plasma aldosterone concentration (PAC), plasma angiotensin II (Ang II), plasma atrial natriuretic peptide (ANP), serum urea nitrogen (UN), and serum creatinine (Cre) at the end of salt loading (14 weeks of age). (H) Immunohistochemistry of NHE3 and SGLT1 in the jejunum and ileum at the end of salt loading (14 weeks of age). Relative staining intensity for the respective proteins in the positive epithelial cells is shown in the graphs. Gray line and gray box: tap water; black line and black box: salt; \*P < 0.05, \*\*P < 0.01 vs. tap; n = 8 per group.



**Figure S3. Effects of salt loading for 8 weeks in Dahl salt-sensitive rats.** (A) Systolic BP. (B) Body weight. (C) Total body water examined by impedance method. (D) Water and food consumption. (E) Urinary volume and stool weight. (F) Urinary and fecal sodium excretion. (G) Plasma renin activity (PRA), plasma aldosterone concentration (PAC), plasma angiotensin II (Ang II), plasma atrial natriuretic peptide (ANP), serum urea nitrogen (UN), and serum creatinine (Cre) at the end of salt loading (14 weeks of age). (H) Immunohistochemistry of NHE3 and SGLT1 in the jejunum and ileum at the end of salt loading (14 weeks of age). Relative staining intensity for the respective proteins in positive epithelial cells is shown in the graphs. Gray line and gray box: tap; black line and black box: salt; \*P < 0.05; \*\*P < 0.01 vs. tap; n = 8 per group.

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**Figure S4. The expression of NHE3 is unchanged by aldosterone in the Caco-2 human intestinal epithelial cells.** (A) Relative mRNA expression of NHE3 and SGLT1 after the administration of aldosterone (10-10, 10-9, and 10-8 mol/L) for 24 hours. \*: P < 0.05, \*\*: P < 0.01 vs. control in each group. PCR results are expressed as the ratio of mRNA/18S mRNA. n = 4 independent experiments per condition.



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