

# Activation of the intestinal tissue renin-angiotensin system by transient sodium loading in salt-sensitive rats

Masaki Ryuzaki, Kazutoshi Miyashita, Masaaki Sato, Hiroyuki Inoue, Kentaro Fujii, Aika Hagiwara, Asuka Uto, Sho Endo, Takuma Oshida, Kenichiro Kinouchi, and Hiroshi Itoh

**Background:** The renal tissue renin-angiotensin system is known to be activated by salt loading in salt-sensitive rats; however, the response in other organs remains unclear.

**Method:** Spontaneously hypertensive rats were subjected to normal tap water or transient high-salt-concentration water from 6 to 14 weeks of age and were thereafter given normal tap water. From 18 to 20 weeks of age, rats given water with a high salt concentration were treated with an angiotensin II type 1 receptor blocker, valsartan.

**Results:** Sustained blood pressure elevation by transient salt loading coincided with a persistent decrease in the fecal sodium content and sustained excess of the circulating volume in spontaneously hypertensive rats. Administration of valsartan sustainably reduced the blood pressure and normalized the fecal sodium levels. Notably, transient salt loading persistently induced the intestinal tissue renin-angiotensin system and enhanced sodium transporter expression exclusively in the small intestine of salt-sensitive rats, suggesting the potential connection of intestinal sodium absorption to salt sensitivity.

**Conclusion:** These results reveal the previously unappreciated contribution of the intestinal tissue renin-angiotensin system to sodium homeostasis and blood pressure regulation in the pathophysiology of salt-sensitive hypertension.

**Keywords:** intestine, salt absorption, salt-sensitive hypertension, sodium/hydrogen exchanger, tissue renin-angiotensin system

**Abbreviations:** ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; AGT, angiotensinogen; Ang II, angiotensin II; ANP, atrial natriuretic peptide; ARB, angiotensin II type 1 receptor blocker; AT1R, angiotensin II type 1 receptor; AT2R, angiotensin II type 2 receptor; BP, blood pressure; CKD, chronic kidney disease; Cre, creatinine; DS rats, Dahl salt-sensitive rats; ENaC $\beta$ ,  $\beta$  subunit of the epithelial sodium channel ENaC; HS, high-salt concentration water; MasR, Mas receptor; NaCl, sodium chloride; NHE3, sodium/hydrogen exchanger isoform 3; NT, normal tap water; PAC, plasma aldosterone concentration; PRA, plasma renin activity; RAS, renin-angiotensin system; ROS, reactive oxygen species; SGLT1, sodium glucose cotransporter 1;

SHRs, spontaneously hypertensive rats; UN, urea nitrogen; WKY, Wistar Kyoto

## INTRODUCTION

Hypertension causes various health problems, including cardiovascular and renal diseases. Although hypertension is attributed to numerous predisposing factors, such as obesity and diabetes, sodium intake predominantly impacts blood pressure (BP) regulation [1]. Hence, dietary salt restriction and the use of diuretics remain the cornerstones of antihypertensive therapy in several guidelines [2,3]. However, current guidelines may not be very effective as the number of individuals with hypertension (caused by excessive salt intake) is still rising [4,5]. Therefore, targeting the regulation of sodium handling can be a novel therapeutic approach for hypertension.

The renal regulation of urinary sodium excretion and its relevance to hypertension have been extensively studied [6,7]. However, very few studies have focused on whether intestinal sodium absorption contributes to the pathogenesis of hypertension. The sodium/hydrogen exchanger isoform 3 (NHE3) is a major absorptive sodium transporter in the intestine [8,9]. In fact, NHE3 inhibitors reduce BP in rat models of hypertension and chronic kidney disease (CKD), and one of these NHE3 inhibitors has become clinically available for patients with CKD [10]. Interestingly, hormonal control of electrolyte transporters in the intestinal epithelial cells is implicated in

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Division of Endocrinology, Metabolism, and Nephrology, Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan

Correspondence to Kazutoshi Miyashita, MD, PhD, Division of Endocrinology, Metabolism, and Nephrology, Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. Tel: +81 3 5363 3878; fax: +81 3 3359 2745; e-mail: miyakaz@z6.keio.jp

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extracellular volume regulation [11]. Among those hormones, the renin-angiotensin system (RAS) appears to impact intestinal sodium absorption [12,13]. The components of RAS are expressed in the gut [13,14]; therefore, it is conceivable that the alteration of intestinal local RAS is linked to the pathophysiology of hypertension through regulation of sodium absorption in the gut.

We previously reported the ‘salt memory’ effect, in which persistent hypertension is induced by transient salt loading in rat models of salt-sensitive hypertension, such as spontaneously hypertensive rats (SHRs) and Dahl salt-sensitive rats [6,15]. The medial hypertrophy of renal arterioles, impaired glomerular perfusion, and increased synthesis of renin are driven by transient salt administration and could be responsible for the underlying mechanisms of persistent hypertension [6,16]. Moreover, transient treatment with high-dose angiotensin II (Ang II) type 1 receptor (AT1R) blocker (ARB) is able to provide beneficial effects, including prolonged reduction of BP and regression of renal arteriolar hypertrophy in SHRs and Dahl salt-sensitive rats [17–20]. These findings suggest that the activation of the RAS because of impaired glomerular perfusion may partly mediate sustained BP elevation after transient salt loading. However, little is known about the sodium balance, including the changes of sodium excretion in the urine or dietary sodium absorption from the gut, in the ‘salt memory’ state.

Here, we demonstrate that a prolonged increase in intestinal sodium absorption and sustained excess of circulating volume seems to contribute to persistent BP elevation after transient salt loading. Intestinal local RAS appeared to be continuously activated during and after salt loading, likely leading to increased sodium absorption through the overexpression of NHE3 in the small intestine. Supporting this notion, the pharmacological reduction of the intestinal local RAS activity by short-term, high-dose administration of a RAS inhibitor attenuated salt memory effects in SHRs. Importantly, intestinal local RAS was also activated in Dahl salt-sensitive rats by salt loading, albeit limited in salt-resistant Wistar Kyoto (WKY) rats as compared with that in salt-sensitive SHRs or Dahl salt-sensitive rats. These observations reveal previously unappreciated functions of intestinal local RAS on sodium homeostasis and provide new insights into the pathophysiology of salt-sensitive hypertension.

## MATERIALS AND METHODS

### Animals

#### Experiment 1

To investigate how sodium levels and fluid volume are altered in response to salt intake and to seek the impacts of sodium handling in the gut and the kidney, 6-week-old male SHRs (body weight approximately 130 g) were randomly divided into the following three groups ( $n = 16$  per group): normal tap water (NT); high salt-concentration water (HS) [1% (wt/vol) NaCl]; and valsartan (ARB) administration in HS rats. HS rats were treated with high salt-concentration (1% NaCl) drinking water from 6 to 14 weeks of age, followed by normal tap water until the end of the experiment. From 18 to 20 weeks of age, HS rats were fed a normal-salt diet containing ARB.

SBP and body weight were recorded every 2 weeks, while food and water consumption, volume and sodium excretion of urine and stools, and total body water amount were examined every 4 weeks in each group of rats. At the end of the experiment (when rats reached 28 weeks of age), and additionally at 14, 15, and 21 weeks of age ( $n = 16$  per group, per age) to investigate the changes immediately after the HS or ARB treatment, the rats were euthanized by rapid decapitation to collect their blood and tissue samples, including the kidney, jejunum, ileum, proximal colon, and distal colon. Each organ was flash frozen in liquid nitrogen apart from tissues for immunohistochemistry that were immersed and fixed in 4% paraformaldehyde before being embedded in paraffin.

#### Experiment 2

To investigate the response to salt loading in different rat models, 6-week-old male WKY or DS rats were randomly divided into the following two groups ( $n = 8$  per group): NT and HS. HS rats were treated with high salt-concentration (1% NaCl) drinking water from 6 to 14 weeks of age. Biological parameters were measured according to the method in experiment 1, and (at 14 weeks of age) the rats were euthanized to collect their blood and tissue samples.

Further details of the experiments (including animal selection, assays, RNA extraction, real-time quantitative PCR analysis, analysis of intestinal and renal histological features, and cell culture) are provided in the online-only Data Supplement, <http://links.lww.com/HJH/B738>.

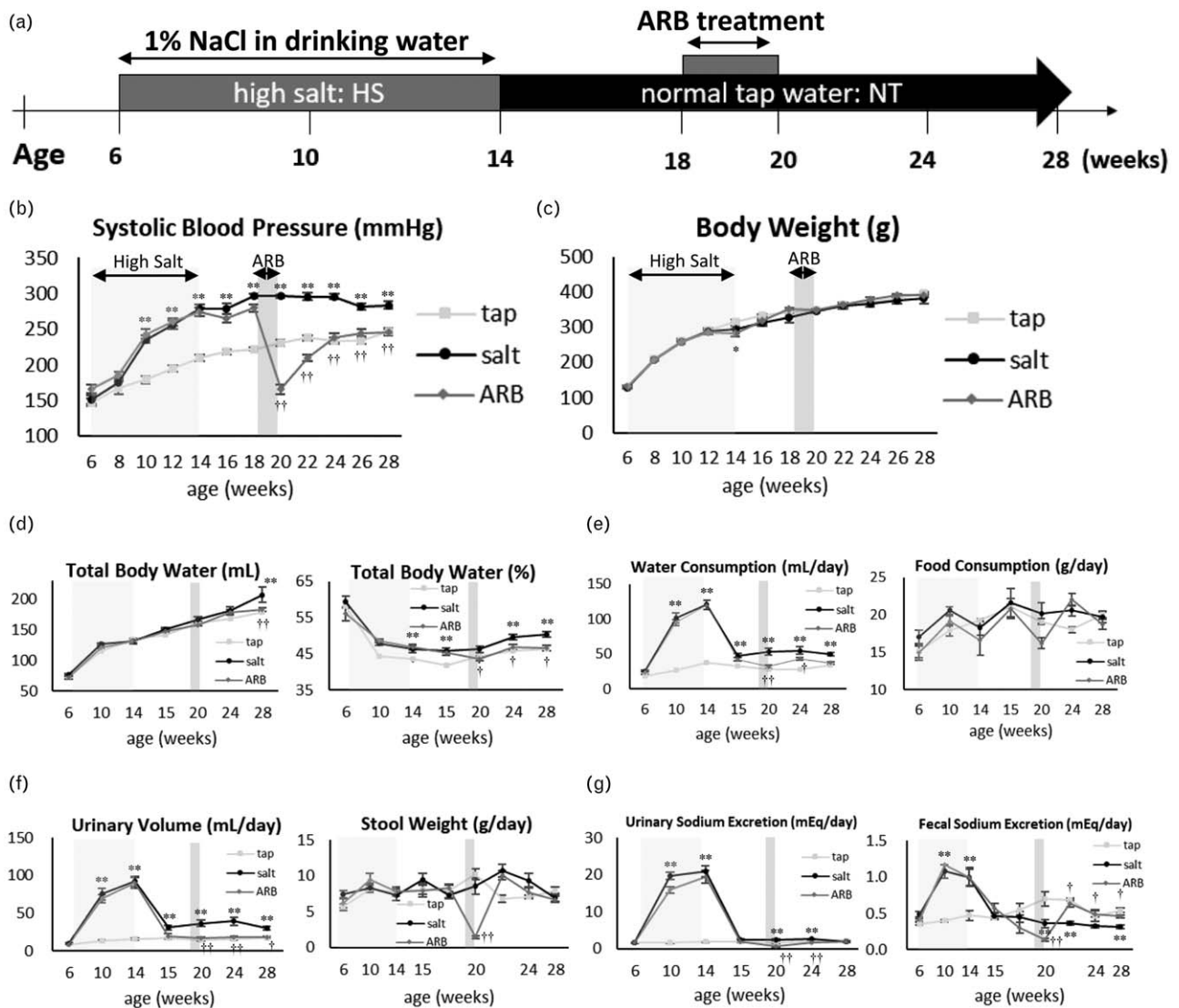
#### Statistical analyses

All the data are presented as mean  $\pm$  standard error of mean values. The mean value of two groups were compared using Student's *t* test. Results obtained over time were analyzed using two-way ANOVA with Bonferroni post hoc test, and differences in the mean values of more than three groups were statistically analyzed using one-way ANOVA and Tukey's post hoc test. (GraphPad Prism 8: GraphPad Software, San Diego, California, USA). *P* less than 0.05 was considered statistically significant.

## RESULTS

### Sustained elevation of blood pressure and reduction of fecal sodium content after transient salt loading, which was regressed by short-term blockade of renin-angiotensin system, in spontaneously hypertensive rats

SHRs were given either NT or HS for 8 weeks, followed by NT for 14 weeks (Fig. 1a). HS rats were treated with an ARB for 2 weeks (Fig. 1a). SBP was elevated after salt loading and sustained over the resumption of NT (Fig. 1b). ARB administration dramatically lowered BP even after the end of the treatment (Fig. 1b). The amount and proportion of total body water remained higher over the return to NT treatment in HS rats, despite the similar body weight, food intake, and stool quantity between HS and NT rats (Fig. 1c–f). In parallel, the increase in water consumption and urinary volume induced by HS were sustained after the end of HS treatment (Fig. 1e and f). Intriguingly, urinary sodium excretion from HS rats remained high, while fecal



**FIGURE 1** Administration of angiotensin II type 1 receptor blocker abolishes the sustained elevation of SBP and the decreased fecal sodium content after transient salt loading in SHRs. (a) Schematic presentation of study design. Tap: rats given normal tap water (NT). Salt: rats transiently given high salt concentration (1% NaCl) drinking water (HS). ARB: HS rats transiently treated with valsartan. HS rats were given salt in drinking water from 6 to 14 weeks of age, and were subsequently treated with tap water. From 18 to 20 weeks of age, the HS rats were treated with valsartan (ARB). (b) SBP. (c) Body weight. (d) Total body water examined with an impedance method. (e) Water and food consumption. (f) Urinary volume and stool weight. (g) Urinary and fecal sodium excretion. Light gray line with a square: tap; black line with a circle: salt; dark gray line with a rhomboid: ARB. \**P* less than 0.05; \*\**P* less than 0.01 salt vs. tap; †*P* less than 0.05; ††*P* less than 0.01 ARB vs. salt; *n* = 16 per group. Light gray vertical columns: high salt concentration water treatment period; dark gray vertical columns: ARB treatment period. ARB, angiotensin II type 1 receptor blocker.

sodium content was suppressed as compared with that in NT rats after the transient salt loading (Fig. 1g, Fig. S1, <http://links.lww.com/HJH/B738>, and Table 1). ARB treatment persistently reduced the total body water amount,

water consumption, and urinary volume, despite comparable body weight, food consumption, and stool weight except over the period of ARB administration (Fig. 1c–f). Importantly, ARB treatment increased fecal sodium content

**TABLE 1. Transient salt loading for 8 weeks causes a sustained increase in the sodium absorption**

Sodium balance (/mouse)	14 weeks			21 weeks			28 weeks		
	Tap	Salt	ARB	Tap	Salt	ARB	Tap	Salt	ARB
Total intake (mEq/day)	2.97 ± 0.04	23.39 ± 0.19**	23.01 ± 0.39**	2.94 ± 0.22	3.57 ± 0.08	2.90 ± 0.07††	3.09 ± 0.07	3.03 ± 0.11	2.88 ± 0.11
Fecal excretion (mEq/day)	0.44 ± 0.07	1.00 ± 0.13**	1.06 ± 0.12**	0.70 ± 0.10	0.36 ± 0.02**	0.64 ± 0.05†	0.54 ± 0.03	0.32 ± 0.02**	0.46 ± 0.02†
Urinary excretion (mEq/day)	1.85 ± 0.05	20.86 ± 1.51**	20.56 ± 1.81**	1.64 ± 0.13	2.24 ± 0.11**	1.76 ± 0.05††	2.11 ± 0.06	1.83 ± 0.13	1.84 ± 0.08
Estimated absorption [mEq/day (%intake)]	0.68 (22.8%)	1.54 (6.6%)	1.38 (6.0%)	0.60 (20.3%)	0.97 (27.2%)	0.50 (17.3%)	0.44 (14.3%)	0.88 (29.0%)	0.58 (20.1%)

Time-dependent shift of sodium balance in spontaneously hypertensive rats at 14, 21, and 28 weeks of age. Estimated absorption was calculated by subtracting the total amount of sodium output (in urine and feces) from the mean of total sodium intake. Data were taken from the experiments summarized in Fig. 1. For each of the rat groups (tap, salt, ARB) at each age (14, 21, and 28 weeks of age), the total intake, fecal excretion, and urinary excretion of sodium were compared with other rat groups. \*\**P* less than 0.05; \**P* less than 0.01 vs. tap; ††*P* less than 0.05; †*P* less than 0.01 ARB vs. salt; *n* = 16 per group. ARB, angiotensin II type 1 receptor blocker.

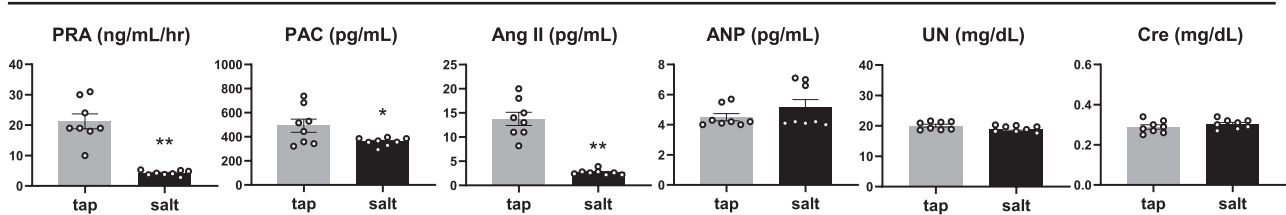
and decreased the urinary sodium excretion as compared with the HS rats without RAS inhibition (Fig. 1g, Fig. S1, <http://links.lww.com/HJH/B738>, and Table 1).

### Activation of systemic renin-angiotensin system by transient salt loading in rats with salt-induced hypertension

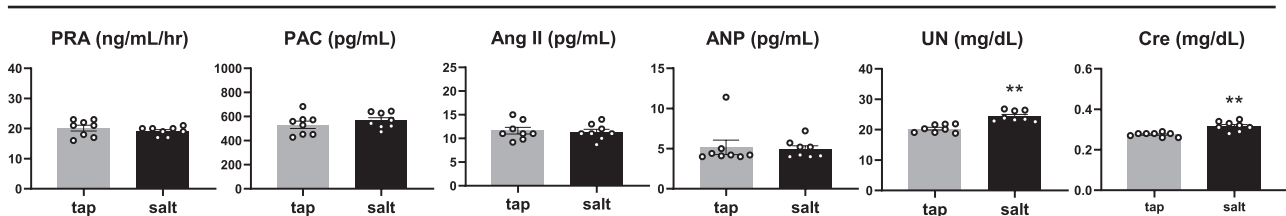
Levels of RAS components, such as plasma renin activity (PRA), plasma aldosterone concentration (PAC), and plasma Ang II,

were markedly suppressed at the end of the salt-loading period (at 14 weeks of age), suggesting the predictable inhibition of systemic RAS by sodium overload (Fig. 2a). Plasma levels of atrial natriuretic peptide (ANP) as well as serum levels of urea nitrogen and creatinine (Cre) from HS rats were comparable with those from NT rats (Fig. 2a). At 15 weeks of age, 1 week after the end of the salt-loading period, plasma levels of RAS components were normalized, whereas the serum UN and Cre levels were elevated in HS rats (Fig. 2b). At 28 weeks of age, plasma levels of RAS components and ANP, along with serum

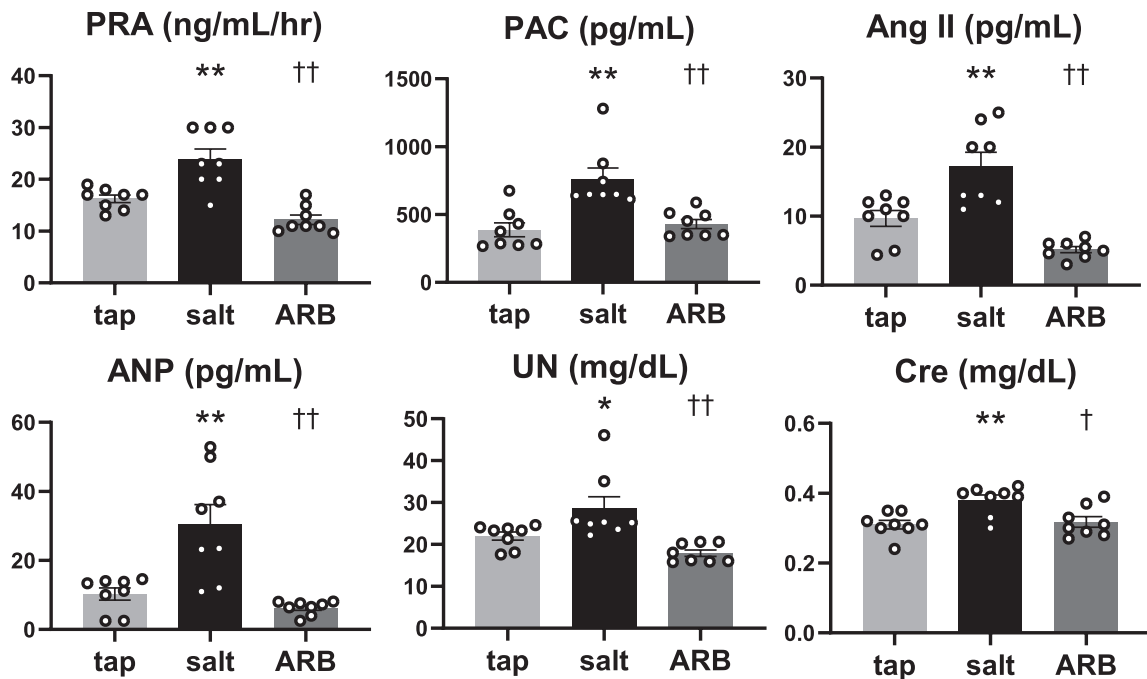
#### (a) 14 weeks of age (during salt loading)



#### (b) 15 weeks of age (tap water)



#### (c) 28 weeks of age (tap water)



**FIGURE 2** Pulse treatment of angiotensin II type 1 receptor blocker inhibits the circulating renin-angiotensin system activation after transient salt loading. (a) 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). (b) PRA, PAC, plasma Ang II, plasma ANP, serum UN, and serum Cre 1 week after the end of salt loading (15 weeks of age). (c) PRA, PAC, plasma Ang II, plasma ANP, serum UN, and serum Cre at 14 weeks after the end of salt loading (28 weeks of age). \*\**P* less than 0.05; \**P* less than 0.01 salt vs. tap; †*P* less than 0.05; ††*P* less than 0.01 ARB vs. salt; *n* = 8 per group. ARB, angiotensin II type 1 receptor blocker.

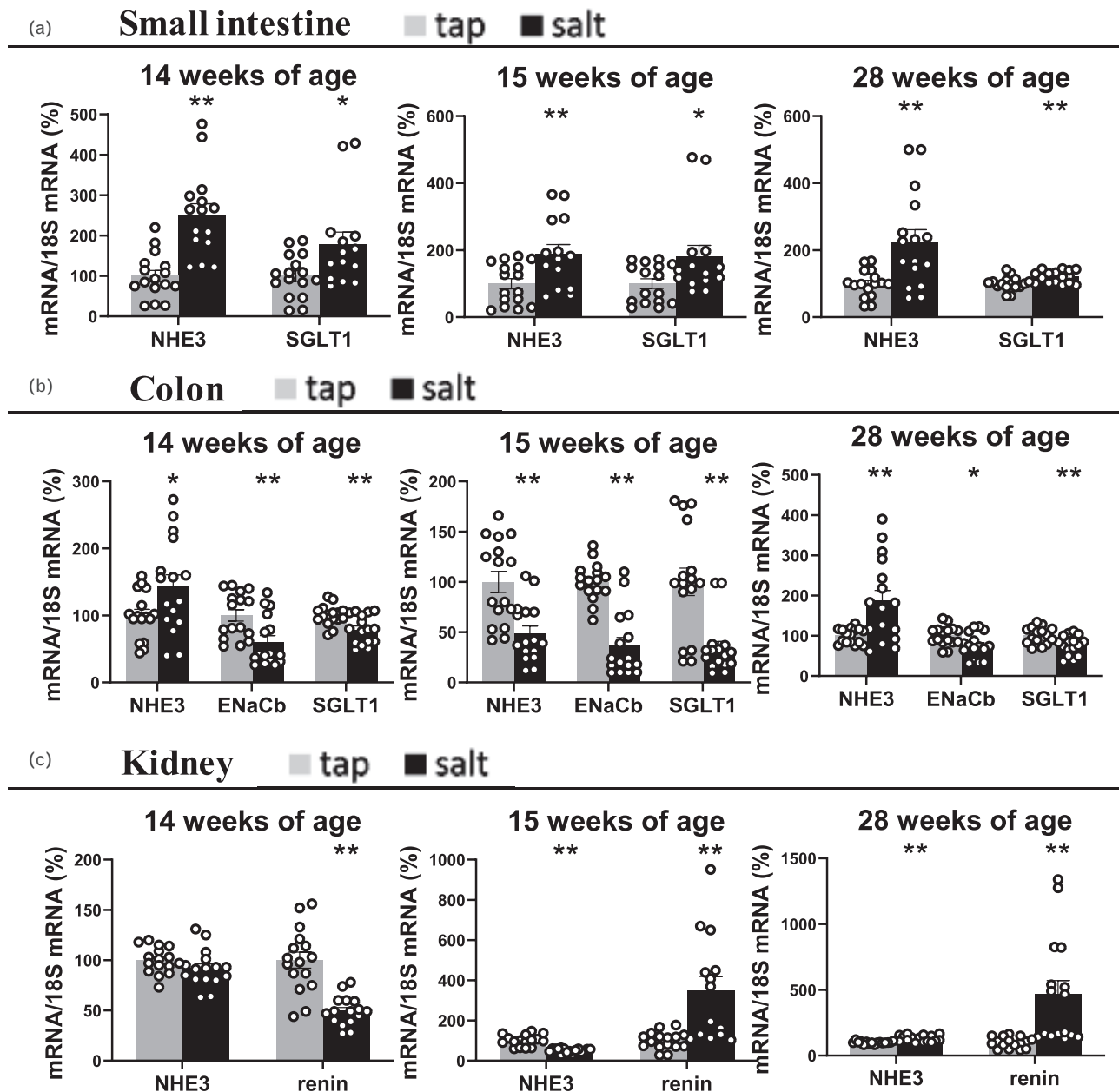


levels of UN and Cre, were increased in HS rats as compared with that in NT rats (Fig. 2c). In contrast, such changes were reversed with ARB treatment (Fig. 2c).

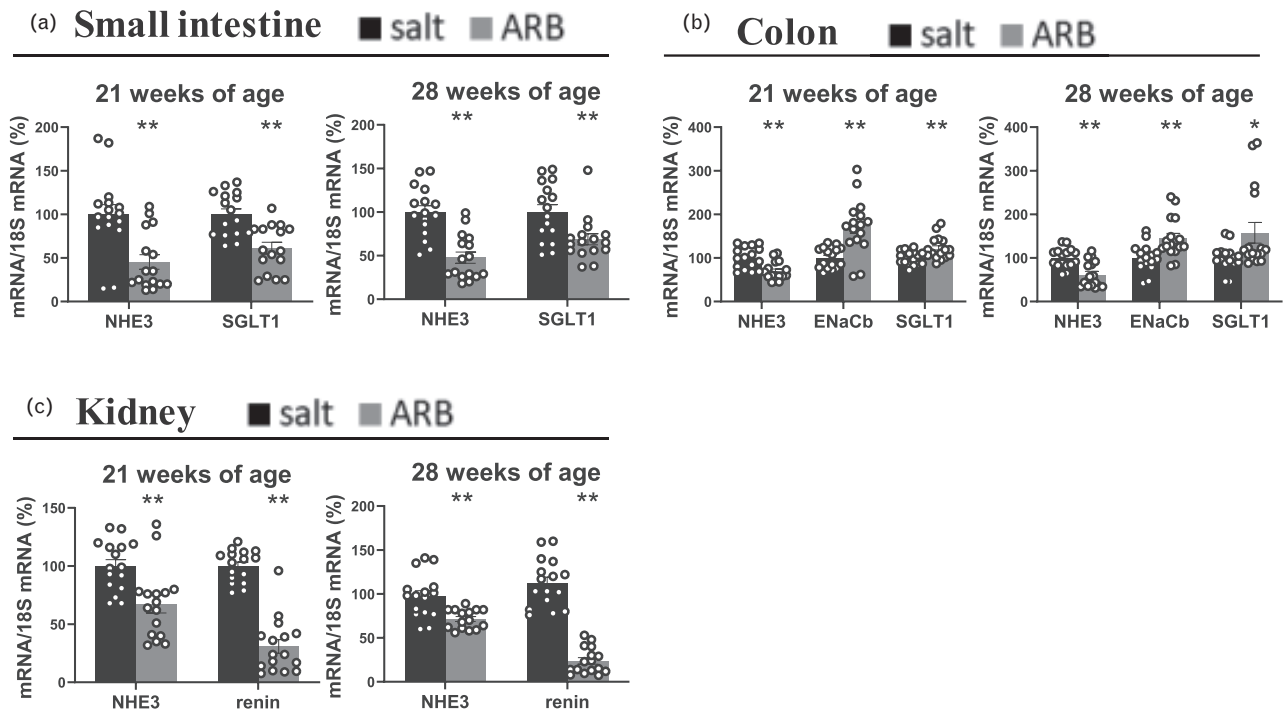
### Intestinal and renal genomic response of sodium transporters to salt loading relies on renin-angiotensin system

Expression of gene encoding for NHE3 was induced in both, the small intestine and the colon, from HS rats at the end of the salt-loading period (14 weeks of age; Fig. 3a and b). *NHE3* gene expression was consistently upregulated in the small

intestine during the return to NT, while being temporarily repressed in the colon 1 week after the end of HS treatment (15 and 28 weeks of age; Fig. 3a and b). Salt loading also caused sustained upregulation of gene encoding for sodium glucose cotransporter 1 (SGLT1) in the small intestine (Fig. 3a). In contrast, colonic expression of genes encoding for SGLT1 and the  $\beta$  subunit of the epithelial sodium channel ENaC (ENaCb) were both repressed by temporary sodium administration over the experimental period (Fig. 3b). In contrast, the expression of renal NHE3 was similar between the HS and NT rats at the end of the salt-loading period;



**FIGURE 3** Transient salt loading for 8 weeks causes sustained elevation of sodium–hydrogen exchanger isoform 3 and sodium glucose cotransporter 1 mRNA expression in the small intestine. (a) Relative sodium–hydrogen exchanger isoform 3 (NHE3) and sodium glucose cotransporter 1 (SGLT1) mRNA expression in the small intestine at the end of salt loading (14 weeks of age), 1 week after salt loading (15 weeks of age), and 14 weeks after the end of salt loading (28 weeks of age). (b) Relative NHE3, epithelial sodium channel  $\beta$  (ENaCb), and SGLT1 mRNA expression in the colon at the end of salt loading (14 weeks of age), 1 week after salt loading (15 weeks of age), and 14 weeks after the end of salt loading (28 weeks of age). (c) Relative NHE3 and renin mRNA expression in the kidney at the end of salt loading (14 weeks of age), 1 week after salt loading (15 weeks of age), and 14 weeks after the end of salt loading (28 weeks of age). Gray box: tap; black box: salt. The results are expressed as the ratio of mRNA/18S mRNA. \**P* less than 0.05; \*\**P* less than 0.01 vs. tap; *n* = 16 per group.



**FIGURE 4** Pulse treatment of angiotensin II type 1 receptor blocker inhibits the elevated expression of sodium–hydrogen exchanger isoform 3 and sodium glucose cotransporter 1 after transient salt loading in the small intestine. (a) Relative NHE3 and SGLT1 mRNA expression in the small intestine at 7 and 14 weeks after the end of transient salt loading (21 and 28 weeks of age, respectively). (b) Relative NHE3, ENaCb, and SGLT1 mRNA expression in the colon at 7 and 14 weeks after the end of transient salt loading (21 and 28 weeks of age, respectively). (c) Relative NHE3 and renin mRNA expression in the kidney at 7 and 14 weeks after the end of transient salt loading (21 and 28 weeks of age, respectively). Black box: salt; dark gray box: ARB. The results are expressed as the ratio of mRNA/18S mRNA. \**P* less than 0.05; \*\**P* less than 0.01 vs. salt; *n* = 16 per group. ARB, angiotensin II type 1 receptor blocker; NHE3, sodium–hydrogen exchanger isoform 3; SGLT1, sodium glucose cotransporter 1.

however, the expression was induced in HS rats 14 weeks after the end of the salt-loading period (Fig. 3c). Renal renin gene expression was repressed by salt loading, and it was markedly induced after the resumption of tap water treatment (Fig. 3c).

Conversely, NHE3 gene expression was repressed by ARB treatment in the small intestine, colon, and kidney of HS rats (Fig. 4a–c). The SGLT1 expression was also reduced by ARB treatment in the small intestine (Fig. 4a), whereas being induced in the colon (Fig. 4b). Additionally, ENaCb expression was increased by ARB treatment in the colon (Fig. 4b). Renal renin expression was significantly inhibited by ARB treatment in HS rats (Fig. 4c).

In line with gene expression, both NHE3 and SGLT1 protein staining intensity was consistently induced in both, the jejunum and ileum after the end of salt loading, and such induction was abrogated by RAS inhibition (Fig. 5a and b). Vascular wall medial thickening was noted in the renal arterioles taken from HS rats 14 weeks after the end of salt administration (Fig. 5c). ARB attenuated medial thickening of the renal arterioles, consistent with the suppression of PRA by ARB treatment (Figs. 2c and 5c).

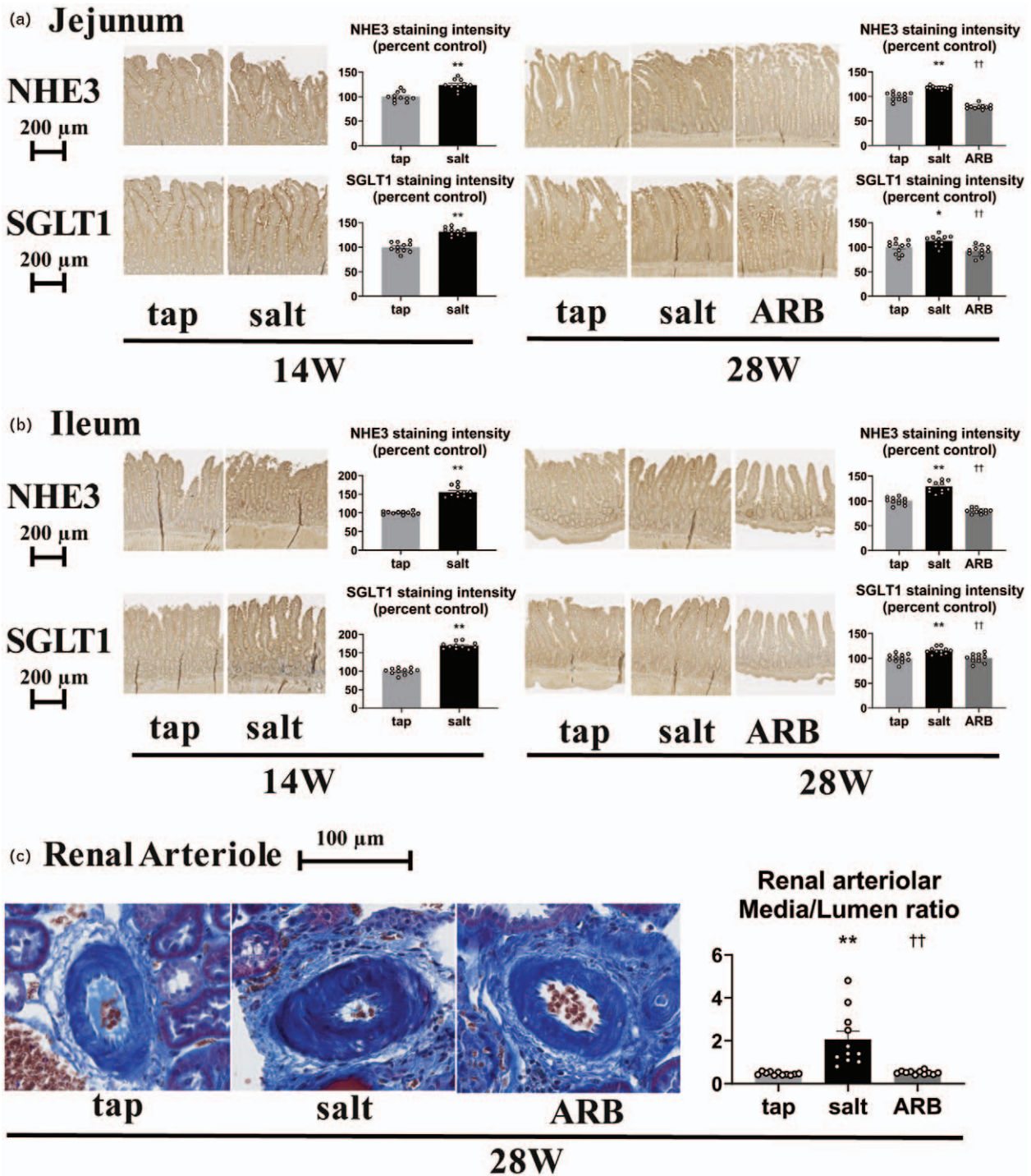
### Intestinal local renin-angiotensin system is activated in spontaneously hypertensive rats by transient salt loading

Local RAS components in the gut were explored in SHR (Fig. 6a–d). Transient administration of sodium significantly elevated the Ang II levels in both, the jejunum and ileum (14 weeks of age; Fig. 6a and c), and the increase

in Ang II was further pronounced 14 weeks after the end of salt administration (28 weeks of age; Fig. 6a and c). Expression of genes encoding for angiotensinogen (AGT), angiotensin-converting enzyme (ACE), AT1R, and Ang II type 2 receptor (AT2R) in both, the jejunum and ileum was induced by salt loading (14 weeks of age; Fig. 6b and d), and the gene induction was partially maintained over 14 weeks after the end of salt administration (28 weeks of age; Fig. 6b and d). ARB treatment repressed intestinal Ang II levels as well as expression of genes encoding for AGT, ACE, AT1R, and AT2R (28 weeks of age; Fig. 6a–d). Expression of genes encoding for angiotensin-converting enzyme 2 (ACE2) and Mas receptor (MasR) showed mostly an opposite response to other RAS components against salt loading and RAS inhibition (Fig. 6b and d).

### Potential connection of intestinal local renin-angiotensin system to salt-sensitivity

WKY and DS rats were subjected to 8 weeks of salt loading (14 weeks of age; Fig. 7a–f, Figs. S2a–h, <http://links.lww.com/HJH/B738>, and S3a–h, <http://links.lww.com/HJH/B738>). SBP and the proportion of total body water were unchanged upon HS administration in WKY rats, while a significant rise was noted in DS rats (Figs. S2a, <http://links.lww.com/HJH/B738> and S3a, <http://links.lww.com/HJH/B738>). In line with SHRs, HS treatment in WKY and DS rats induced elevation of the water consumption, urinary volume, and urinary and fecal sodium excretion, despite almost similar body weight, food intake, and stool quantity between HS and NT rats (Figures S2b–f, <http://links.lww.com/HJH/B738> and

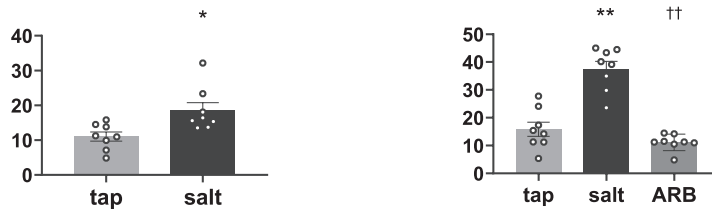


**FIGURE 5** Pulse treatment of angiotensin II type 1 receptor blocker inhibits the sustained upregulation of sodium–hydrogen exchanger isoform 3 and sodium glucose cotransporter 1 expression in the small intestine and the medial thickening of renal arterioles after transient salt loading for 8 weeks. (a) Immunohistochemistry of NHE3 and SGLT1 in the jejunum at the end of salt loading (14 weeks of age) and 14 weeks after the end of salt loading (28 weeks of age). Relative staining intensity for the respective proteins in positive epithelial cells is shown in the graphs. (b) Immunohistochemistry of NHE3 and SGLT1 in the ileum at the end of salt loading (14 weeks of age) and 14 weeks after the end of salt loading (28 weeks of age). Relative staining intensity for the respective proteins in positive epithelial cells is shown in the graphs. (c) Representative photomicrographs of renal arterioles at 14 weeks after the end of salt loading (28 weeks of age). The renal arteriolar media/lumen ratio is shown in the graphs. \*\**P* less than 0.05; \**P* less than 0.01 salt vs. tap; ††*P* less than 0.05; †*P* less than 0.01 ARB vs. salt; *n* = 11 per group. NHE3, sodium–hydrogen exchanger isoform 3; SGLT1, sodium glucose cotransporter 1.

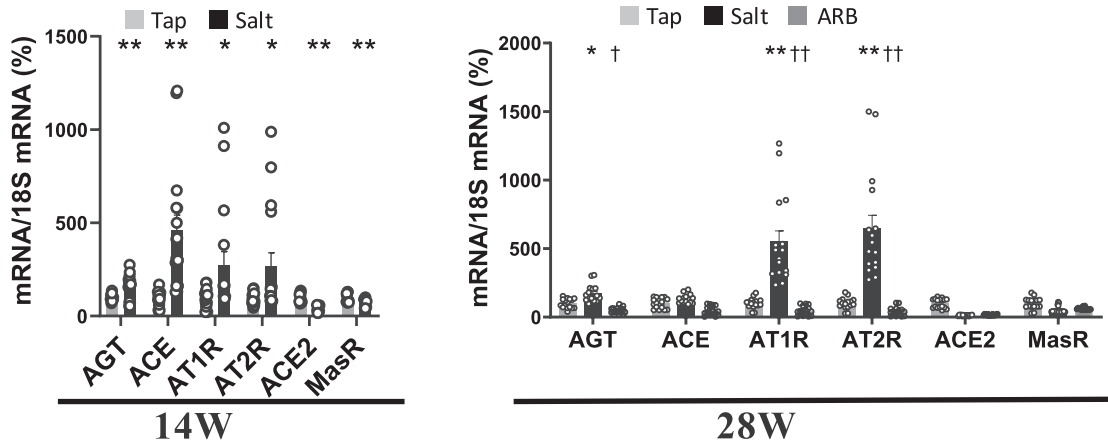
S3b–f, <http://links.lww.com/HJH/B738>). Plasma levels of RAS components (PRA, PAC, and Ang II) were suppressed by HS administration in both, WKY and DS rats (Figs. S2g, <http://links.lww.com/HJH/B738> and S3g, <http://links.lww.com/HJH/B738>).

Interestingly, the serum levels of UN were only increased in DS rats with HS administration (Fig. 2a, Figs. S2g, <http://links.lww.com/HJH/B738>, and S3g, <http://links.lww.com/HJH/B738>).

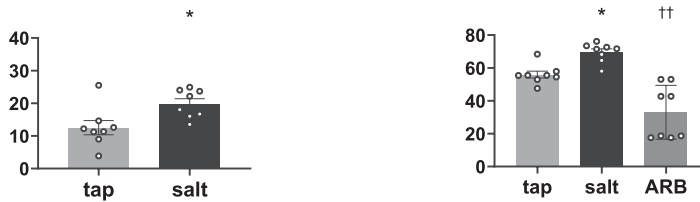
(a) **Jejunum AngII (pg/g of tissue)**



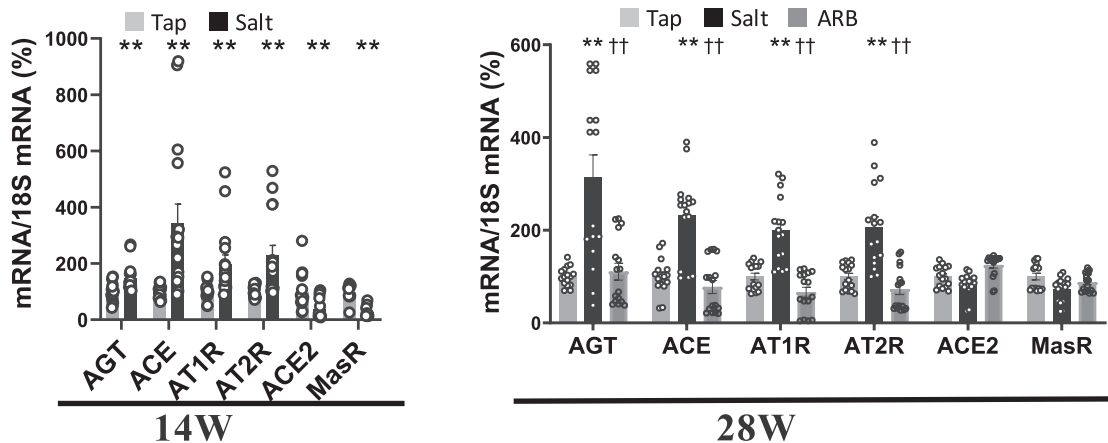
(b) **Gene expression**



(c) **Ileum AngII (pg/g of tissue)**

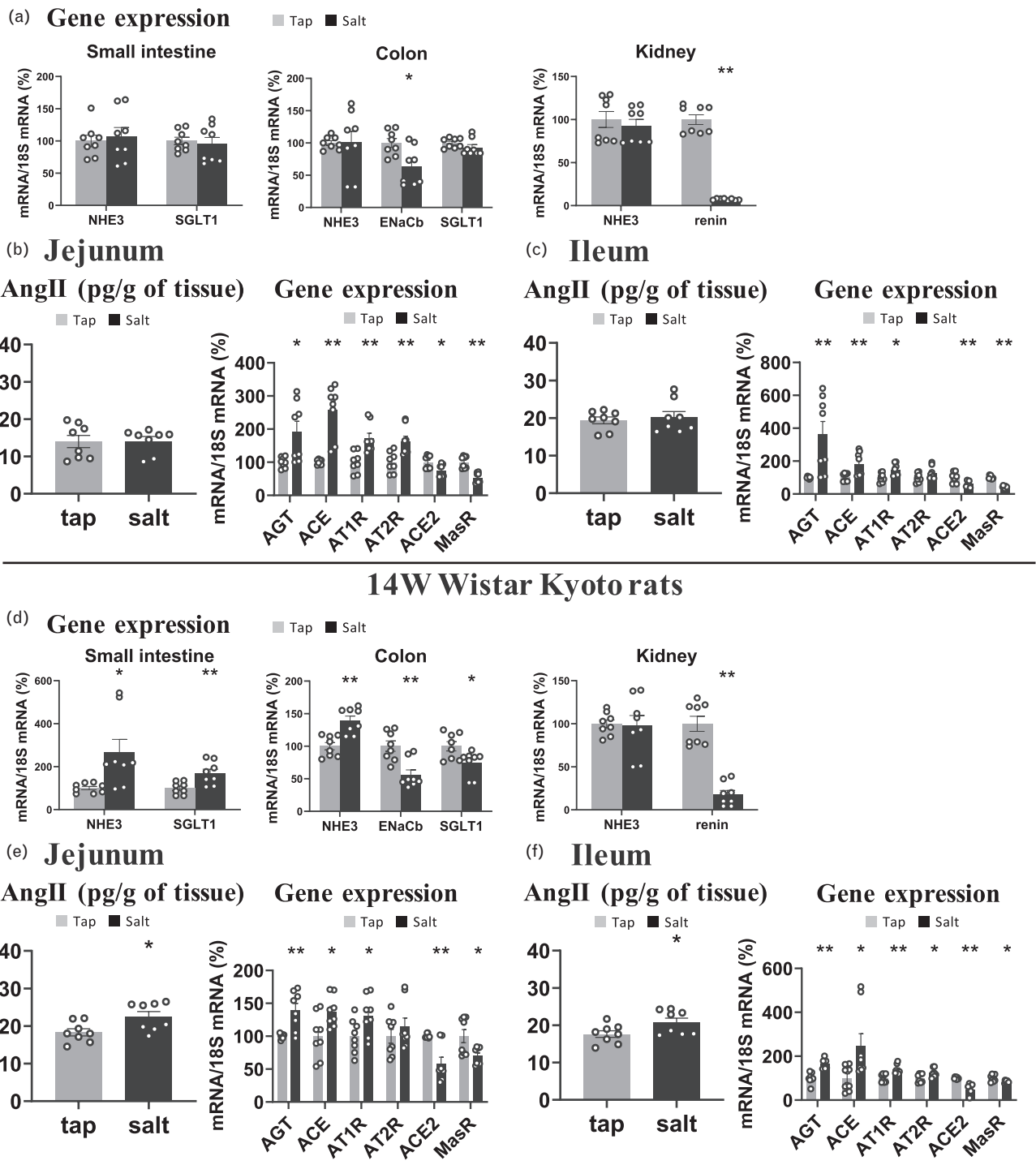


(d) **Gene expression**



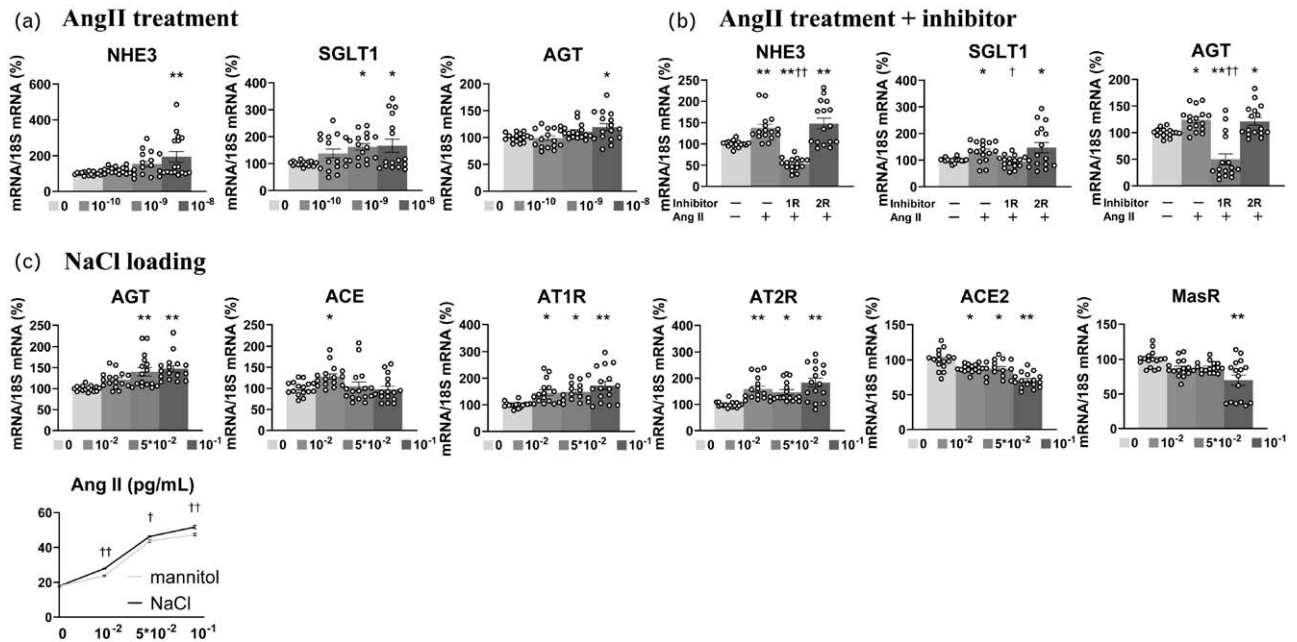
**FIGURE 6** Pulse treatment of angiotensin II type 1 receptor blocker inhibits the sustained activation of intestinal tissue renin-angiotensin system after transient salt loading for 8 weeks. (a) The intestinal tissue Ang II concentration in the jejunum at the end of salt loading (14 weeks of age) and 14 weeks after the end of salt loading (28 weeks of age). (b) The relative mRNA expression of angiotensinogen (AGT), angiotensin-converting enzyme (ACE), Ang II type 1 receptor (AT1R), Ang II type 2 receptor (AT2R), angiotensin-converting enzyme 2 (ACE2), and Mas receptor (MasR) in the jejunum at the end of salt loading (14 weeks of age) and 14 weeks after the end of salt loading (28 weeks of age). The results are expressed as the ratio of mRNA/18S mRNA. (c) The intestinal tissue Ang II concentration in the ileum at the end of salt loading (14 weeks of age) and 14 weeks after the end of salt loading (28 weeks of age). (d) The relative mRNA expression of AGT, ACE, AT1R, AT2R, ACE2, and MasR in the ileum at the end of salt loading (14 weeks of age) and 14 weeks after the end of salt loading (28 weeks of age). The results are expressed as the ratio of mRNA/18S mRNA. Light gray box: tap; black box: salt; dark gray box: ARB; \*\**P* less than 0.05; \**P* less than 0.01 salt vs. tap; ††*P* less than 0.05; †*P* less than 0.01 ARB vs. salt; *n* = 8 per group in Ang II concentration measurement; *n* = 16 per group in mRNA expression measurement.





**14W Dahl salt-sensitive rats**

**FIGURE 7** Salt loading for 8 weeks causes significant activation of intestinal tissue renin-angiotensin system in Dahl salt-sensitive rats, but insignificant activation in Wistar Kyoto rats. (a) Relative NHE3 and SGLT1 mRNA expression in the small intestine, NHE3, ENaCb, and SGLT1 mRNA expression in the colon, and NHE3 and renin mRNA expression in the kidney of Wistar Kyoto rats (WKY) at the end of salt loading (14 weeks of age). (b) The intestinal tissue Ang II concentration and the relative mRNA expression of AGT, ACE, AT1R, AT2R, ACE2, and MasR in the jejunum of WKY at the end of salt loading (14 weeks of age). (c) The intestinal tissue Ang II concentration and the relative mRNA expression of AGT, ACE, AT1R, AT2R, ACE2, and MasR in the ileum of WKY at the end of salt loading (14 weeks of age). (d) Relative NHE3 and SGLT1 mRNA expression in the small intestine, NHE3, ENaCb, and SGLT1 mRNA expression in the colon, and NHE3 and renin mRNA expression in the kidney of Dahl salt-sensitive rats (DS rats) at the end of salt loading (14 weeks of age). (e) The intestinal tissue Ang II concentration and the relative mRNA expression of AGT, ACE, AT1R, AT2R, ACE2, and MasR in the jejunum of DS rats at the end of salt loading (14 weeks of age). (f) The intestinal tissue Ang II concentration and the relative mRNA expression of AGT, ACE, AT1R, AT2R, ACE2, and MasR in the ileum of DS rats at the end of salt loading (14 weeks of age). Gray box: tap; black box: salt. PCR results are expressed as the ratio of mRNA/18S mRNA. \**P* less than 0.05; \*\**P* less than 0.01 vs. tap; *n* = 8 per group. ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; AGT, angiotensinogen; AngII, angiotensin II; AT1R, angiotensin II type 1 receptor; AT2R, angiotensin II type 2 receptor; MasR, Mas receptor; NHE3, sodium/hydrogen exchanger isoform 3; SGLT1, sodium glucose cotransporter 1.



**FIGURE 8** Ang II increases the expression of sodium–hydrogen exchanger isoform 3 and sodium glucose cotransporter 1 in Caco-2 human intestinal epithelial cells. (a) The relative mRNA expression of NHE3, SGLT1, and AGT after the administration of Ang II (10<sup>-10</sup>, 10<sup>-9</sup>, and 10<sup>-8</sup> mol/l) for 24 h. (b) The relative mRNA expression of NHE3, SGLT1, and AGT after the administration of Ang II (10<sup>-8</sup> mol/l) in combination with valsartan (AT1R blocker, 10<sup>-5</sup> mol/l) or PD123319 (AT2R blocker, 10<sup>-5</sup> mol/l) for 24 h. \**P* less than 0.05, \*\**P* less than 0.01 vs. control in each group, †*P* less than 0.05, ††: *P* less than 0.01 vs. the treated group without inhibitors. (c) The relative mRNA expression of AGT, ACE, AT1R, AT2R, ACE2, and MasR, and the concentration of Ang II in the cultured media after the administration of NaCl (10<sup>-2</sup>, 5 × 10<sup>-2</sup>, and 10<sup>-1</sup> mol/l) for 24 h in comparison to that treated with mannitol with the same osmotic pressure as NaCl. Gray line: mannitol; black line: NaCl; \**P* less than 0.05; \*\**P* less than 0.01 vs. no NaCl administration group; †*P* less than 0.05; ††: *P* less than 0.01 vs. mannitol-treated group. PCR results are expressed as the ratio of mRNA/18S mRNA. *n* = 4 independent experiments per condition. ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; AGT, angiotensinogen; AngII, angiotensin II; AT1R, angiotensin II type 1 receptor; AT2R, angiotensin II type 2 receptor; MasR, Mas receptor; NHE3, sodium/hydrogen exchanger isoform 3; SGLT1, sodium glucose cotransporter 1.

Contrary to SHR, *NHE3* and *SGLT1* gene expressions were unchanged in both, the small intestine and colon by salt loading in WKY rats, while being induced in DS rats (Fig. 3a and b and Fig. 7a and d). Genomic response of other sodium transporters to salt loading displayed a similar tendency in WKY and DS rats as compared with that in SHR (Fig. 3a–c and Fig. 7a and d). In line with the gene expression, intestinal NHE3 and SGLT1 protein staining intensity was increased only in DS rats at the end of salt loading (Figs. S2h, <http://links.lww.com/HJH/B738> and S3h, <http://links.lww.com/HJH/B738>). Likewise, significant elevation of intestinal Ang II by salt loading in SHR was recapitulated only in DS rats, but not in WKY (Fig. 6a and c and Fig. 7b, c, e, and f). Meanwhile, intestinal expression of genes encoding for AGT, ACE, AT1R, and AT2R was induced, whereas the expression of genes encoding for ACE2 and MasR was repressed with salt loading in both, WKY and DS rats (Fig. 7b, c, e, and f).

### Intestinal renin-angiotensin system induces the gene expression of sodium transporters via the angiotensin II– Ang II type 1 receptor axis

Caco-2 human intestinal epithelial cells were treated with Ang II, sodium chloride (NaCl), or aldosterone (Fig. 8a–c and Fig. S4a, <http://links.lww.com/HJH/B738>). Expression of genes encoding for NHE3, SGLT1, and AGT was increased with Ang II treatment (Fig. 8a). The increased expression of *NHE3*, *SGLT1*, and *AGT* genes was significantly repressed by an AT1R inhibitor, valsartan, but not by

an AT2R inhibitor, PD123319 (Fig. 8b). It is noteworthy that the expression of genes encoding for RAS components (apart from ACE2 and MasR) as well as Ang II concentration in the cultured media, were increased with NaCl treatment (Fig. 8c). Aldosterone failed to induce the gene expression of sodium transporters (Figure S4a, <http://links.lww.com/HJH/B738>).

## DISCUSSION

The present study identified how sustained BP elevation with transient salt loading coincided with a persistent decrease in the fecal sodium content and sustained excess of circulating volume in SHR. Salt-induced hypertension and the acceleration of intestinal sodium absorption appeared to be reversed with RAS inhibition. Transient salt loading and treatment with ARB seemed to continuously impact renal excretion and intestinal absorption of sodium, indicating the involvement of RAS in ‘salt memory’ effects. Systemic RAS was suppressed during salt loading but was eventually activated by impaired glomerular perfusion, based on renal arteriolar hypertrophy and the prominent induction of renal *renin* gene expression. Intriguingly, intestinal tissue RAS was induced even from the period of salt loading, independent of systemic RAS. Intestinal sodium transporter expression was increased by salt loading, likely driven by RAS activation, suggesting a relationship to augmented intestinal sodium absorption. Salt-induced intestinal RAS activation was also observed in

DS rats but only partially in WKY rats, indicating the potential connection of intestinal RAS to salt sensitivity.

The intestine engages in the digestion and absorption of dietary nutrients. Importantly, almost all sodium and fluids are absorbed through the small intestine (~95%), and the remainder is absorbed via the colon (~4%) [11]. With regard to the molecular regulation of intestinal NaCl absorption, NHE3 is a major sodium transporter and is predominantly expressed in the apical brush border membrane of the intestinal epithelium in addition to the renal proximal tubule [11]. In the renal proximal tubules, NHE3 reabsorbs up to 75% of the sodium [21]. In the human intestine, NHE3 is expressed at a higher level in the ileum and jejunum than in the colon [22]. NHE3-deficient mice (NHE3<sup>-/-</sup>) display moderate salt wasting from the digestive system with diarrhea and reduced BP [23,24]. Conversely, unlike nontransgenic mice, NHE3<sup>-/-</sup> mice with transgenic expression of NHE3 in the small intestine (tgNHE3<sup>-/-</sup>) are resistant to chronic volume depletion, low BP, and dietary salt deficiency [25,26]. Furthermore, an NHE3 inhibitor (which possesses low oral bioavailability and acts exclusively on the gut) increases the fecal sodium content, decreases the urinary sodium excretion, and lowers BP in SHR rats [27].

On the basis of these observations, NHE3 in the gut is presumably a key regulator for the homeostasis of sodium balance and BP. In addition to NHE3, SGLT1 in the small intestine and the colon as well as ENaCb in the colon are known to impinge on intestinal sodium absorption. Consistent with our observations, a high-salt diet increases the expression of intestinal SGLT1 [28]. However, the pharmacological inhibition of SGLT1 does not induce severe diarrhea [29]. Furthermore, SGLT1<sup>-/-</sup> mice have near-normal BP [30]. Thus, SGLT1 is likely to have a marginal impact on intestinal sodium absorption. Meanwhile, colon-specific ENaCb-deficient mice exhibit increased fecal sodium excretion [31]. Our studies indicated that the genomic response to salt loading in the small intestine and colon exhibits a distinct signature. More precisely, the intestinal expression of NHE3 and SGLT1 was persistently upregulated after transient salt loading, while ENaCb was downregulated as a reflection. Accordingly, it is conceivable that augmented sodium absorption because of increased expression of sodium transporters (especially in the small intestine) is involved in the mechanism of 'salt memory'.

Intestinal sodium and water absorption are stimulated by Ang II [32]. Although multiple mechanisms control the NHE3 expression [33], Ang II activates NHE3 through AT1R-dependent mechanisms in the kidney and cultured intestinal epithelial cells [34]. Supporting these previous studies, our in-vitro experiments demonstrated that the expression of intestinal NHE3 was augmented by the Ang II–AT1R pathway. Thus, the intestinal Ang II–AT1R pathway likely contributes to the establishment of 'salt memory', whereas the Ang II–AT1R blockade may contribute to the regression of 'salt memory', through regulation of the expression of sodium transporters in the gut.

Local RAS is found in many tissues and is known to be regulated differently from systemic RAS. As for the reaction against salt loading, a high-salt diet causes upregulation of the tissue RAS components in the heart and kidney of DS rats [35,36]. In specific subsets of salt-sensitive rats, the

regulation of intrarenal RAS seems to be independent of circulating RAS; renin dictates the Ang II levels in the plasma, whereas AGT is likely to be a critical regulator of Ang II production in the kidney [37]. A high-salt diet induces intrarenal AGT, promoting the generation of renal Ang II, which, in turn, may further stimulate the local production of AGT, thereby creating a vicious cycle of intrarenal local RAS activation [37–39]. RAS components have been detected in the intestine, and, strikingly, the concentration of Ang II in the small intestine may be higher than that in the kidney [40]. However, the pathophysiological roles of intestinal local RAS on BP regulation remain unclear [13]. AT1R agonism may increase colonic sodium absorption in rats with CKD [41]. On the contrary, intestinal tissue RAS can be repressed by the administration of ARB [42]. In the present study, RAS components in the small intestine were upregulated with salt loading (especially in salt-sensitive rats), while circulating RAS was suppressed. More precisely, ACE–Ang II–AT1R axis was induced, whereas ACE2–MasR axis, which acts in an opposite manner than the ACE–Ang II–AT1R axis, was suppressed in the small intestine with salt loading. Given that intestinal Ang II was not augmented with salt loading in WKY rats as in SHR rats or DS rats, intestinal RAS may be linked to salt sensitivity. Furthermore, in-vitro experiments have demonstrated that *AGT* gene expression was upregulated after both, Ang II and NaCl treatment in human intestinal epithelial cells. Hence, we propose that NaCl may directly or indirectly activate local RAS in the intestine, thereby contributing to initial or even sustained BP elevation upon salt loading. Other molecules, such as reactive oxygen species (ROS), which activate AGT expression in rat kidney during a high-salt diet [37,38], might also be involved in the intestinal RAS activation. Future studies are warranted to address this question.

Other than the AGT–Ang II vicious cycle *in situ*, sustained elevation of intestinal Ang II after transient salt loading in SHR rats may also be explained by continuously high levels of circulating renin owing to medial thickening of the renal arterioles. In general, increased sodium intake leads to RAS suppression. In fact, our results showed reduction in the renal *renin* gene expression consistent with suppressed plasma renin activity during salt loading. However, in specific subsets of salt-sensitive rats, including SHR rats and DS rats, intrarenal RAS gets induced by salt loading [35,36]; we believe that this leads to renal arteriolar hypertrophy and eventual acceleration of systemic RAS. From the point of view of sodium retention, although ARB reduced urinary sodium excretion, total body water was significantly reduced in the ARB group than in the HS group. Accordingly, the reduction of urinary sodium excretion would be a reflective of the decrease in the absorption of sodium from the intestine. Collectively, owing to RAS attenuation with ARB treatment, BP, circulating volume, and intestinal sodium absorption were significantly reduced, associated with similar regression of renal arteriolar hypertrophy and reduction in the plasma renin activity.

From a clinical perspective, a direct association between intestinal local RAS and hypertension has not been established to date; however, some hypotheses can be proposed based on our results. In some subsets of hypertensive



patients, for example, obese hypertensive patients, systemic RAS is reported to be inappropriately normal, or even accelerated despite higher sodium intake [43,44]. Importantly, obesity-related hypertension is known to exhibit the pattern of salt-sensitive hypertension [45] and be modulated by the gastrointestinal tract; obesity-related hypertension can be effectively managed with metabolic surgery [46]. Especially, elderly subjects, African Americans, Asians, and obese patients are thought to be salt-sensitive [47,48]. One hallmark of salt sensitivity is the inability to appropriately suppress renal local RAS in response to salt loading [49], and an ARB was shown to improve the salt sensitivity presumably through the inhibition of local RAS [50]. Moreover, the 'memory' effect of antihypertensive treatment with an ARB but not with a calcium channel blocker, was observed in humans [51], maybe through the modification of tissue RAS. Although the precise regulation remains unclear as most previous studies on salt-sensitive hypertension were mainly focused on renal regulation, our results suggest the relation of salt-sensitive hypertension to intestinal local RAS.

In conclusion, we demonstrated that the activation of intestinal local RAS by transient salt loading was sustained in salt-sensitive rats, likely contributing to enhanced sodium transporter expression in the small intestine. Possible augmentation of intestinal sodium absorption caused the increased circulating volume and persistent BP elevation. Notably, transient salt loading induced intestinal local RAS in the gut of salt-sensitive rats, SHR and DS rats; but incompletely in salt-resistant WKY rats. Given that the effects of diuretics in humans are influenced by renal function and that the adherence to a low-sodium diet is generally not accomplished easily, the concept of limiting intestinal sodium absorption could be a promising strategy in the development of a new type of antihypertensive therapy. In fact, our findings provide important insights into the understanding of the intestinal mechanism of 'salt memory' and salt-sensitive hypertension.

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## Conflicts of interest

There are no conflicts of interest.

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