Depressor effect of chymase inhibitor in mice with high salt-induced moderate hypertension

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Devarajan S, Yahiro E, Uehara Y, Habe S, Nishiyama A, Miura S, Saku K, Urata H. Depressor effect of chymase inhibitor in mice with high salt-induced moderate hypertension. Am J Physiol Heart Circ Physiol 309: H1987–H1996, 2015. First published October 2, 2015; doi:10.1152/ajpheart.00721.2014.—The aim of the present study was to determine whether long-term high salt intake in the drinking water induces hypertension in wild-type (WT) mice and whether a chymase inhibitor or other antihypertensive drugs could reverse the increase of blood pressure. Eight-week-old male WT mice were supplied with drinking water containing 2% salt for 12 wk (high-salt group) or high-salt drinking water plus an oral chymase inhibitor (TPC-806) at four different doses (25, 50, 75, or 100 mg/kg), captopril (75 mg/kg), losartan (100 mg/kg), hydrochlorothiazide (3 mg/kg), eplerenone (200 mg/kg), or amlodipine (6 mg/kg). Control groups were given normal water with or without the chymase inhibitor. Blood pressure and heart rate gradually showed a significant increase in the high-salt group, whereas a dose-dependent depressor effect of the chymase inhibitor was observed. There was also partial improvement of hypertension in the losartan- and eplerenone-treated groups but not in the captopril-, hydrochlorothiazide-, and amlodipine-treated groups. A high salt load significantly increased chymase-dependent ANG II-forming activity in the alimentary tract. In addition, the relative contribution of chymase to ANG II formation, but not actual average activity, showed a significant increase in skin and skeletal muscle, whereas angiotensin-converting enzyme-dependent ANG II-forming activity and its relative contribution were reduced by high salt intake. Plasma and urinary renin-angiotensin system components were significantly increased in the high-salt group but were significantly suppressed in the chymase inhibitor-treated group. In conclusion, 2% salt water drinking for 12 wk caused moderate hypertension and activated the renin-angiotensin system in WT mice. A chymase inhibitor suppressed both the elevation of blood pressure and heart rate, indicating a definite involvement of chymase in salt-sensitive hypertension.

mouse; high salt; hypertension; renin-angiotensin system; chymase inhibitor

The association between high salt (HS) intake and elevated blood pressure has been well established by cross-sectional and longitudinal epidemiological studies. Observational studies and clinical trials performed in the general population have indicated that a higher or lower salt intake is associated with an increase or a decrease of blood pressure, respectively (20, 32). However, the response of blood pressure to changes in salt intake varies considerably from one individual to another, which is a phenomenon known as “salt sensitivity.” An epidemiological study (53) has indicated that ~51% of hypertensive patients and 26% of normotensive subjects show salt sensitivity. It has been reported that a variety of mutations and polymorphisms of genes related to Na⁺ channels are involved in salt sensitivity and result in dysregulation of Na⁺ metabolism. Such mutations include those affecting the synthesis and circulating levels of mineralocorticoids (28) as well as mutations causing renal inflammation, oxidative stress, and an increase of intrarenal ANG II (13, 38). However, previous reports about salt sensitivity have been based on relatively short-term salt loading, and the mutations studied were not common in the general population. Approximately one-quarter of the adult population has hypertension, and it is due to long-term excess salt intake in the majority rather than short-term abnormalities of Na⁺ handling. Therefore, it is important to clarify the mechanisms involved in long-term salt-induced hypertension.

Changes in plasma and tissue components of the renin-angiotensin system (RAS) after salt loading are currently under debate because recent studies in Dahl salt-sensitive rats, an established model of salt-sensitive hypertension, have suggested that salt-sensitive hypertension is accompanied by an increase of cardiac ANG II (18, 47) or renal angiotensinogen (23), whereas standard textbook theory predicts a decrease. In fact, it has been a general consensus that both plasma and tissue RAS activity are suppressed by short-term salt loading (7, 24, 45).

Long-term HS intake increases blood pressure in normotensive pigs (8), baboons (6), African green monkeys (42), and chimpanzees (9). In addition, it has been documented that a high-salt diet causes hypertension in rodents, such as Dahl salt-sensitive rats and DOCA-salt-sensitive rats (18, 47), but not in normotensive wild-type (WT) mice. Establishing a salt-sensitive mouse model of hypertension would allow us to study the role of chymase in salt-dependent hypertension because WT mice carry human type α-chymase (mouse mast cell chymase-4 and -5).
Most of the known chymases exist in two forms, α and β (5). Chymase is a chymotrypsin-like serine protease that is common to several species, which include primates, dogs, sheep, and hamsters. Humans have a single α-chymase gene, whereas rats and mice not only have the α-chymase gene (mouse mast cell protease-4 and rat mast cell protease-5, respectively) but also possess up to 14 β-chymase genes (11).

Although treatment with angiotensin-converting enzyme (ACE) inhibitors or ANG II receptor type 1 blockers (ARBs) as monotherapy has been clinically proven to lower blood pressure and provide organ protection, combined use of both these classes of drugs has been suggested to be important for blood pressure normalization (2). Li et al. (27) reported that incomplete suppression of ANG II in mice is due to mouse mast cell protease-4-dependent ANG II formation.

Chymase has been demonstrated to be a potential ANG II-forming enzyme in several organs of humans, hamsters, rats, rabbits, dogs, pigs, and marmosets (1), but no study has assessed chymase-mediated ANG II formation in various organs of the mouse, especially in animals with a HS load. We hypothesized that long-term HS intake is directly associated with elevation of blood pressure through a chymase-mediated mechanism. Therefore, we performed the present study to establish a model of salt-sensitive hypertension in WT mice as well as to investigate the association of chymase with salt-sensitive hypertension and the effects of a chymase inhibitor (CI), RAS inhibitors (captopril and losartan), a thiazide diuretic [hydrochlorothiazide (HCTZ)], a mineralocorticoid receptor antagonist (MRA; eplerenone), and a Ca2+ channel blocker (amlodipine).

**MATERIALS AND METHODS**

**Experiments**

**Experiment I: HS loading protocol and CI treatment.** Eight-week-old male WT mice (C57BL/6J) were fed normal chow and divided into the following seven weight-matched groups that were treated for 12 wk. The nonsalt loading control group was given normal water (NW), the NW + CI (NW-CI) group received normal water plus a CI (100 mg/kg), the HS loading group received HS drinking water containing 2% NaCl, and the HS + CI (HS-CI) groups received HS drinking water plus different doses of a CI (25, 50, 75, or 100 mg/kg TPC-806, provided by Teijin Pharma) (52). Blood pressure was measured in conscious animals at baseline (week 0) and then weekly for 12 wk. Body weight was also measured in week 0 and then weekly together with food and water intake. Mice were placed in metabolic cages in week 0 and at the end of week 12 for 24-h urine collection. Urine samples were stored at −80°C until analysis. After scarification, several mouse organs were harvested and stored at −80°C until used for the ANG II-forming activity assay.

**Experiment II: effect of a CI on established hypertension after HS loading for 12 wk.** Eight-week-old male WT mice (C57BL/6J) were bred as described above and divided into three groups. The NW group received NW from week 8 to week 28, and the HS group received water containing 2% NaCl for the same period. In addition, the HS-CI group received HS water for the same period, and oral treatment with a CI (TPC-806 at 100 mg·kg−1·day−1) was performed from week 12 to week 20. Blood pressure, heart rate, and body weight were measured at baseline and every 4 wk up to 12 wk and followed by weekly measurement for the following 8 wk.

**Experiment III: effect of RAS blockers, a diuretic, a MRA, and a Ca2+ channel blocker on HS-induced moderate hypertension.** Eight-week-old male WT mice (C57BL/6J) were divided into seven groups and treated for 12 wk. The NW group received NW, and HS groups received water containing 2% NaCl, HS water plus captopril (75 mg/kg orally, Sigma-Aldrich) (3), losartan (50 mg/kg orally, LKT laboratories) (22), HCTZ (3 mg/kg, Sigma-Aldrich) (35), eplerenone (200 mg/kg, a kind gift from Pfizer) (51), or amlodipine (6 mg/kg, a kind gift from Pfizer) (34). Blood pressure, heart rate, and body weight were measured at the beginning of the experiment and weekly up to week 12.

All mice used in experiments I–III were housed in a temperature- and humidity-controlled room with a 12:12-h light-dark cycle. Experimental protocols and animal care methods were approved by Experimental Animal Committee at Fukuoka University and performed according to their guidelines.

**Measurements of Blood Pressure and Heart Rate**

Blood pressure and heart rate were measured noninvasively using a computerized tail-cuff system (MK 2000, Muromachi Kikai). Mice were acclimatized to the apparatus by performing daily measurement sessions for 5 days at 1 wk before the start of each experiment. Six pressure and heart rate readings were recorded each time, and the average value was used for analysis. To avoid diurnal variations of blood pressure, all measurements were made by the same person at the same time of day (9–12 AM).

**Measurements of Plasma Angiotensinogen and Aldosterone**

After preparation of plasma samples according to the manufacturer’s instructions, plasma levels of angiotensinogen and aldosterone were measured with enzyme immunoassay kits (Immuno-Biological Laboratories, Gunma, Japan, for angiotensinogen and Assay Designs, Ann Arbor, MI, for aldosterone).

**Measurement of Plasma Renin Activity**

Plasma renin activity (PRA) was determined by a luminescent immunoassay with a kit purchased from Alpco Diagnostics (Salem, NH). The immunoassay quantified the amount of ANG I that was enzymatically generated by renin from its substrate angiotensinogen.

**Assessment of Tissue ANG II-Forming Activity**

The formation of ANG II from ANG I (ANG II-forming activity) was determined as described elsewhere with some modifications (1). To measure both ACE- and chymase-dependent ANG II-forming activity, the particulate fraction from each tissue was prepared and incubated with synthetic ANG I (200 mmol/l) with or without inhibitors for the appropriate time at 37°C. ANG II was then detected by reverse-phase HPLC (Waters) using a C18 reverse-phase HPLC column (2.2 × 25 cm, Vydac) with a linear acetonitrile gradient (3–13% over 15 min) in 25 mmol/l triethylamine-phosphate buffer (pH 3) at a flow rate of 2 ml/min. The retention times of synthetic His-Leu, ANG I (5–10), ANG II, and ANG I were 1.7, 8.8, 9.4, and 12.3 min, respectively, whereas the other peaks (2.0, 8.4, 11.0, 11.5, 14.6, and 15.4 min) were not identified. ANG II-forming activity was expressed as picograms of ANG II formed per minute per milligram of protein. Captopril (100 μmol/l, Sigma Chemical)-inhibitable ANG II formation (in mmol/l) or chymostatin (50 μmol/l, Sigma Chemical)-inhibitable and aprotinin (200 μmol/l, Bayer)-insensitive ANG II formation (in mmol/l) were determined as ACE- or chymase-dependent activity, respectively. Analysis of each sample was performed in duplicate, and the reproducibility and quality of all data were confirmed before statistical analysis.

**Assay of Plasma ANG II**

Blood samples were obtained by ocular puncture, and 1 ml blood was added to a chilled tube containing 500 mmol EDTA, 100 mmol captopril, 125 mmol 1,10-phenanthroline, and 1 mmol pepstatin. Plasma was then obtained by centrifugation and stored at −80°C.
Plasma ANG II concentrations were measured by a radioimmunoassay, as previously described (33).

Measurements of Urine Parameters

Urinary excretion of angiotensinogen, aldosterone, albumin, and creatinine was determined with commercial kits (Immuno-Biological Laboratories for angiotensinogen, Assay Designs for aldosterone, Kamiya Biomedical, Seattle, CA, for albumin, and Cayman Chemical, Ann Arbor, MI, for creatinine). Daily excretion of angiotensinogen, aldosterone, albumin, and creatinine was calculated from the volume of urine collected over 24 h in each mouse.

Results are expressed as means ± SD. Statistical analysis was performed with a paired t-test for within-group comparisons or one-way ANOVA for between-group comparisons followed by Tukey’s multiple-comparison least-significant test. P values of <0.05 were considered to indicate statistical significance.

RESULTS

Changes of Body Weight, Food Intake, Water Intake, and Urine Volume

Figure 1 shows changes of body weight (A), food intake (B), water intake (C), and urine volume (D). The baseline body weight of each group was similar, and no significant differences were noted during the study period. Daily food intake also showed no significant differences among the groups throughout the experiment. On the other hand, mice that consumed HS drinking water showed a significant increase of water intake (~5-fold increase, P < 0.0001) and had a significantly larger urine volume (~4-fold, P < 0.0001) compared with control mice that consumed NW.

Increase of Blood Pressure and Heart Rate With HS Loading and Suppression by CI Administration

There were no significant differences of blood pressure or heart rate among the groups at baseline and during week 1 (Fig. 2). There was a significant elevation of systolic blood pressure (Fig. 2A), diastolic blood pressure (Fig. 2B), and heart rate (Fig. 2C) from week 2 to week 12 in the HS group, whereas mice from the 75 and 100 mg·kg CI groups had significantly (P < 0.0001) lower blood pressure throughout the study period. The CI showed dose-dependent antihypertensive and negative chronotropic effects (Fig. 2, A–C), and a dose of 100 mg·kg completely suppressed that elevation of blood pressure so that it was similar to that in the control group without salt loading, whereas the reduction of heart rate was less prominent.

CI Normalizes High Blood Pressure and Markedly Reduces Heart Rate in Mice With Salt-Induced Moderate Hypertension

Salt loading for 12 wk established a moderate elevation of systolic/diastolic blood pressure and heart rate compared with control mice that received NW. Administration of the CI (100 mg·kg) for 8 wk significantly decreased systolic blood pressure (Fig. 3A), diastolic blood pressure (Fig. 3B), and heart rate (Fig. 3C). Significant reductions of heart rate were noted in the CI groups from week 2 to week 12 (Fig. 3C). There were no significant differences of blood pressure or heart rate among the groups at baseline and during week 1 (Fig. 2). There was a significant elevation of systolic blood pressure (Fig. 2A), diastolic blood pressure (Fig. 2B), and heart rate (Fig. 2C) from week 2 to week 12 in the HS group, whereas mice from the 75 and 100 mg·kg CI groups had significantly (P < 0.0001) lower blood pressure throughout the study period. The CI showed dose-dependent antihypertensive and negative chronotropic effects (Fig. 2, A–C), and a dose of 100 mg·kg completely suppressed that elevation of blood pressure so that it was similar to that in the control group without salt loading, whereas the reduction of heart rate was less prominent.

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and heart rate (Fig. 3C). There were no significant differences of body weight among the groups throughout the experimental period.

**Activation of the Plasma RAS and Suppression by CI Administration**

In week 12, the plasma level of angiotensinogen (Fig. 4A) was significantly elevated ($P < 0.0001$) in the HS group, whereas it was significantly lower ($P < 0.0001$) in the CI group compared with the HS group. After 12 wk, PRA was significantly increased ($P < 0.0001$) in the HS group, whereas it was significantly lower ($P < 0.0001$) in the CI group (Fig. 4B). Similarly, plasma levels of ANG II and aldosterone showed a significant increase ($P < 0.0001$) in the HS group, whereas there was a modest but significant ($P < 0.001$) reduction of plasma ANG II and aldosterone in the CI group relative to the HS group (Fig. 4, C and D).

**Increased 24-h Urinary Excretion of Angiotensinogen, Aldosterone, and Albumin With 12-wk Salt Loading and Suppression by CI Administration**

A significant increase in the 24-h urinary excretion of angiotensinogen (Fig. 5A), aldosterone (Fig. 5B), and albumin (5C) was observed in the HS group compared with baseline and with the control group. The CI group showed significantly lower urinary excretion of these parameters compared with the HS group. Urinary excretion of creatinine did not show any statistical differences among the groups (Fig. 5D) before or after HS loading with or without CI treatment.

**Organ-Specific Changes of Chymase-Dependent ANG II-Forming Activity**

As shown in Table 1, there were distinct differences of total ANG II-forming activity among various organs of mice given NW (control data in Table 1). Total ANG II-forming activity was maximal in the alimentary tract followed by the aorta and skin. Other organs, such as the heart, brain, adipose tissue, and skeletal muscle, all showed a similar low level of total ANG II-forming activity. ACE was the dominant ANG II-forming enzyme in the brain, heart, and adipose tissue, whereas chymase was dominant in the alimentary tract, skin, and skeletal muscle. Both enzymes contributed almost equally to ANG II formation in the aorta. After salt loading for 12 wk, total ANG II-forming activity in the alimentary tract showed a significant increase by approximately twofold, whereas total ANG II-forming activity in the skin was significantly lower. There were no significant changes of activity in the brain, heart, aorta, adipose tissue, or skeletal muscle. Baseline total ANG II-forming activity was predominantly chymase dependent in the alimentary tract and the contribution of chymase increased further after 12 wk of salt loading, whereas ACE-dependent ANG II-forming activity showed a significant decrease compared with that in the NW group. A similar increase of chymase-dependent ANG II-forming activity and decrease of
ACE-dependent activity was also found in the skin and skeletal muscle after salt loading.

**Losartan or Eplerenone, But Not Captopril, HCTZ, or Amlodipine, Significantly Ameliorates Salt-Induced Hypertension**

High-dose captopril was unable to reduce the elevation of blood pressure and heart rate induced by a HS load (Fig. 6, A–C), whereas mice that received high-dose losartan or eplerenone showed a significant reduction of systolic blood pressure (Fig. 6A), diastolic blood pressure (Fig. 6B), and heart rate (Fig. 6C). Although the improvement observed with losartan or eplerenone treatment was statistically significant, it should be noted that the actual changes of these parameters were small. There were no significant differences of body weight among the groups (including controls) throughout the study (Fig. 6D).

**DISCUSSION**

Our study was based on the hypothesis that long-term HS intake is directly associated with an elevation of blood pressure via a chymase-mediated mechanism, and it is the first study to demonstrate that HS intake (2% salt in drinking water) induces moderate hypertension in WT mice and activates circulating and urinary RAS components. In previous rodent models, it has been observed that a short-term increase of dietary salt intake does not elevate blood pressure (30, 36), although that addition of other manipulations, such as deoxycorticosterone (41) or ANG II (25), can consistently produce salt-sensitive hypertension. However, no previous study has assessed the impact of prolonged HS loading in WT mice. Among animal models of salt-sensitive hypertension, there are many advantages of using mice, including their striking similarity to humans with respect to anatomy, physiology, and genetic profile (48, 50) and especially the similarity of the circulating and local ANG II-forming systems (40). In the present study, male WT mice were given a HS load (2% NaCl) in their drinking water for 12 wk. There was no significant increase of blood pressure during the first week, consistent with previous reports showing that short-term HS intake does not cause hypertension in mice (30, 49). However, a significant increase of blood pressure was observed from week 2, and it persisted until week 12. Both the gradual increase of blood pressure (from week 0 to week 12) and established hypertension (after 12 wk of HS intake) were completely suppressed by treatment with a CI, indicating that the hypertensive mechanism of this model was chymase dependent.

In addition, we investigated whether oral supplementation with well-characterized captopril, losartan, eplerenone, HCTZ, or amlodipine was effective for suppressing the salt-induced development of moderately high blood pressure. Strikingly, treatment with the ACE inhibitor, diuretics, or amlodipine did not achieve any reduction of blood pressure or heart rate. Although the ARB or eplerenone significantly reduced blood pressure and heart rate, its effect was only partial. These results strongly suggest that an ACE-dependent mechanism was not associated with moderate hypertension in our model, whereas...
the aldosterone cascade and chymase-mediated ANG II formation were involved.

Another important finding of the present study was that plasma and urinary RAS components were not suppressed after 12 wk of salt loading but were significantly elevated. Previous studies have shown that circulating levels of ANG II, aldosterone, and renin (activity or concentration) were all significantly decreased by short-term salt intake in rats (19, 44), whereas chronic salt loading increased circulating ANG II, aldosterone, and PRA in the present study, causing hypertension in normotensive mice by RAS activation. In contrast to our present observations, several studies (30, 49) have found that mice fed a HS diet (8% salt) for up to 4 wk showed no significant change in blood pressure despite consuming ~200 mg salt/day (calculated on the basis of daily food intake). The difference from our results may have several explanations, including a higher salt intake in our study (500 vs. 200 mg/day) and a longer salt loading period (12 vs. 4 wk). In the present study, CI treatment partially, but not completely, reversed the activation of plasma and urinary RAS components. This was in agreement with the partial depressor effect of ARB or eplerenone treatment in our model. Our findings suggest that HS intake induced moderate hypertension in WT mice together with activation of the circulating and urinary RAS, but RAS activation could not fully explain the mechanism of hypertension in our model because ARB or MRA alone could not suppress the elevated blood pressure but CI treatment completely suppressed it.

Therefore, we investigated ANG II-forming systems in various organs of mice with or without long-term HS loading. Our findings suggest that the local ANG II-forming system differed among organs, with the contribution of chymase being important in certain organs, such as the alimentary tract, skin, and skeletal muscle, but not in the brain, heart, aorta, and adipose tissue. Total tissue ANG II-forming activity was highest in the alimentary tract among the organs examined, suggesting that alimentary tract chymase makes the largest contribution to local ANG II formation. In addition, the relative contribution of chymase to ANG II formation in the alimentary tract, skin, and skeletal muscle increased significantly with salt loading, whereas that of ACE decreased in these organs. Therefore, it is plausible that the depressor effect of a CI in our salt-loaded hypertensive model was partially mediated by suppression of chymase-dependent ANG II formation in these organs.

The most striking finding of the present study was that the alimentary tract might play a major role in chymase-mediated induction of moderate hypertension by HS intake. This differs from the concept of chymase playing a major role in cardiovascular tissues, which has been suggested by previous clinical studies (15, 16). Our data showed that HS intake increased total ANG II-forming activity in the alimentary tract by approximately twofold compared with that in mice that received NW and that the increase was mainly due to elevation of chymase-dependent ANG II formation. An increased contribution of chymase to ANG II formation was also found in the skin and skeletal muscle after HS intake, but total ANG II-forming activity did not change in these organs. Therefore, alimentary tract chymase may be primarily responsible for the elevation of blood pressure in the present mouse model.

It has been previously reported that the skin accumulates Na⁺ without proportionate water retention when excess salt is

Fig. 4. Plasma levels of renin-angiotensin system components in week 12. A–D: angiotensinogen (A), plasma renin activity (PRA; B), ANG II (C), and aldosterone (D) in the NW-control (n = 20), HS-control (n = 20), and HS-CI (100 mg·kg⁻¹·day⁻¹, n = 20) groups for 12 wk. Values are means ± SD. #P < 0.0001 vs. the NW-control group; *P < 0.0001 vs. the HS-control group.
present (46). Accumulation of inactive Na\(^+\)/H\(^+\) in the skin has been shown to have many regulatory consequences (29), and therefore the significant increase in the relative contribution of chymase to ANG II formation in the skin after salt loading in our model could have altered cutaneous vascular sensitivity and thus contributed to the increase of blood pressure. Helle et al. reported that 2 wk on a HS diet increased the sensitivity of skin vessels to ANG II, so the relative increase of chymase-dependent ANG II-forming activity with prolonged salt loading in our study may have promoted an increase in the contractility of the cutaneous vasculature.

A HS diet has been shown to increase oxidative stress in skeletal muscle (26) and mesenteric vessels (54). ROS may possibly be involved in the significant increase of the relative contribution of chymase to ANG II formation in skeletal muscle after salt loading. The skin and muscles receive a significant part of total cardiac output (12). Therefore, major changes in the sensitivity of vessels in these organs could have a significant impact on total peripheral resistance and contribute to the increase of blood pressure associated with salt loading.

In the present study, CI treatment significantly reduced heart rate without any differences of body weight, food intake, water intake, or urine volume. An increase of the heart rate with long-term salt loading might represent sympathetic activation, whereas its partial suppression by the CI suggests that chymase is linked to sympathetic activation by a HS load.

It has been previously reported that even short-term oral or central salt loading increases sympathetic activity, especially when salt is directly infused into the brain stem of rats (21, 37). The mechanism by which a HS load activates the sympathetic nervous system has not been completely elucidated, but a recent study (10) has suggested the involvement of oxidative stress. However, there have been no previous reports of an association between chymase and sympathetic activation by salt loading, and this study also provided the first evidence that an oral CI can suppress sympathetic activation. In our study, HS intake did not increase the total or chymase-dependent ANG II-forming activity in the brain, which suggests that chymase has no direct involvement in the sympathetic activation.

Inhibition of the circulating RAS is one of the major mechanisms involved in reducing sympathetic activity, so it seems reasonable to assume that inhibition of circulating RAS components by administration of the CI had a sympatholytic effect in our model. On the other hand, an ACE inhibitor had no effect on blood pressure or heart rate in this model, whereas an ARB or a MRA had a slight (but significant) depressor effect and negative chronotropic effect in the acute phase (weeks 2–4), indicating that chymase-mediated ANG II or aldosterone contributed to the acute phase sympathetic activation to develop moderate hypertension and increased heart rate. RAS or MRA inhibition alone does not explain the effect of the CI. In addition, the CI suppressed all of the RAS components, including PRA, angiotensinogen, and aldosterone, differing from the effects of other RAS inhibitors, such as ACE inhibitors and/or ARBs. This could be an important action of CIs, but the mechanism by which the CI suppressed the entire RAS cascade

Fig. 5. Urinary (U) components of the renin-angiotensin system in week 0 and week 12. A–D: excretion of angiotensinogen (A), aldosterone (B), albumin (C), and creatinine (D) in the NW-control (n = 20), HS-control (n = 20), and HS-CI (100 mg·kg\(^{-1}\)·day\(^{-1}\), n = 20) groups for 12 wk. Data are presented as daily excretion. Values are means ± SD. #P < 0.0001 vs. the NW-control group; *P < 0.0001 vs. the HS-control group.
eplerenone (200 mg·kg⁻¹·day⁻¹) in NW-control mice (Fig. 6). Weekly SBP (A), DBP (B), heart rate (C), and body weight (D) in NW-control mice (n = 20), HS-control mice (n = 20), and mice that receiving HS drinking water plus captopril (75 mg·kg⁻¹·day⁻¹); HS-captopril group; n = 20), losartan (50 mg·kg⁻¹·day⁻¹); HS-losartan group, n = 6), hydrochlorothiazide (3 mg·kg⁻¹·day⁻¹); HS-HCTZ group; n = 6), eplerenone (200 mg·kg⁻¹·day⁻¹); HS-EPL group), or amlodipine (6 mg·kg⁻¹·day⁻¹); HS-amlodipine group) for 12 wk. Points of SBP, DBP, and heart rate data in the HS-HCTZ, HS-captopril, and HS-amlodipine groups did not show significant differences compared with those in the HS-control groups. SBP and DBP in the HS-losartan and HS-EPL groups were significantly lower from week 3 to week 12, whereas heart rates of the same groups were significantly lower from week 2 to week 12 compared with that in the HS-control group.

Table 1. ANG II-forming activity in different organs/tissues

<table>
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<tr>
<th>Organs and Salt Loading Status</th>
<th>Number of Mice/Group</th>
<th>Total ANG II-Forming Activity, pg ANG II·min⁻¹·mg⁻¹</th>
<th>Angiotensin-Converting Enzyme-Dependent ANG II-Forming Activity</th>
<th>% vs. total ANG II-forming activity</th>
<th>Chymase-Dependent ANG II-Forming Activity</th>
<th>% vs. total ANG II-forming activity</th>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>9,517 ± 10,626</td>
<td>900 ± 363</td>
<td>32 ± 3</td>
<td>8,618 ± 10,772</td>
<td>68 ± 33</td>
</tr>
<tr>
<td>HS</td>
<td>8</td>
<td>17,872 ± 14,474*</td>
<td>813 ± 660*</td>
<td>9 ± 11*</td>
<td>17,059 ± 14,055*</td>
<td>91 ± 11*</td>
</tr>
<tr>
<td>Skin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>2,393 ± 494</td>
<td>812 ± 148</td>
<td>34 ± 2</td>
<td>1,581 ± 350</td>
<td>66 ± 2</td>
</tr>
<tr>
<td>HS</td>
<td>10</td>
<td>1,944 ± 533*</td>
<td>526 ± 149*</td>
<td>27 ± 5*</td>
<td>1,419 ± 453</td>
<td>72 ± 5*</td>
</tr>
<tr>
<td>Adipose tissue</td>
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<td></td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>567 ± 237</td>
<td>412 ± 146</td>
<td>74 ± 4</td>
<td>155 ± 92</td>
<td>26 ± 4</td>
</tr>
<tr>
<td>HS</td>
<td>6</td>
<td>413 ± 90</td>
<td>304 ± 74</td>
<td>73 ± 4</td>
<td>108 ± 19</td>
<td>27 ± 4</td>
</tr>
<tr>
<td>Skeletal muscle</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>317 ± 56</td>
<td>135 ± 27</td>
<td>43 ± 7</td>
<td>183 ± 47</td>
<td>57 ± 7</td>
</tr>
<tr>
<td>HS</td>
<td>6</td>
<td>360 ± 123</td>
<td>113 ± 27</td>
<td>33 ± 5*</td>
<td>247 ± 96</td>
<td>67 ± 5*</td>
</tr>
</tbody>
</table>

HS, mice that received high salt loading with 2% NaCl in the drinking water. *P < 0.05 vs. the corresponding control group.

HS, mice that received high salt loading with 2% NaCl in the drinking water. *P < 0.05 vs. the corresponding control group.

Studies of animal models and transgenic mice with increased RAS activity have documented the primary role of angiotensinogen in hypertension (31, 40). It has been previously reported that urinary angiotensinogen and aldosterone levels are better indicators of the severity of organopathy than their plasma levels and that these parameters also play a greater role in the maintenance of hypertension (4). The significant suppression of circulating RAS components by a CI in the present

Fig. 6. Weekly SBP (A), DBP (B), heart rate (C), and body weight (D) in NW-control mice (n = 20), HS-control mice (n = 20), and mice that receiving HS drinking water plus captopril (75 mg·kg⁻¹·day⁻¹); HS-captopril group; n = 20), losartan (50 mg·kg⁻¹·day⁻¹); HS-losartan group, n = 6), hydrochlorothiazide (3 mg·kg⁻¹·day⁻¹); HS-HCTZ group; n = 6), eplerenone (200 mg·kg⁻¹·day⁻¹); HS-EPL group), or amlodipine (6 mg·kg⁻¹·day⁻¹); HS-amlodipine group) for 12 wk. Points of SBP, DBP, and heart rate data in the HS-HCTZ, HS-captopril, and HS-amlodipine groups did not show significant differences compared with those in the HS-control groups. SBP and DBP in the HS-losartan and HS-EPL groups were significantly lower from week 3 to week 12, whereas heart rates of the same groups were significantly lower from week 2 to week 12 compared with that in the HS-control group.
model suggests that such inhibitors could be applicable for treating both hypertension and organopathy. In fact, a reduction of urinary albumin excretion by CI administration was observed in our salt-sensitive hypertensive mice. Thus, the depressor effect of the CI could be related to suppression of both circulating and urinary RAS components in this model of salt-induced hypertension.

Our present data indicate that an ANG II- and aldosterone receptor-mediated mechanism was partially involved in salt-induced moderate hypertension, but this could not explain whole hypertensive mechanism of our mice model, whereas the CI completely suppressed the salt-induced high blood pressure. Therefore, mechanisms other than the renin-angiotensin-aldosterone system, such as endothelin, should be considered because mouse or human chymase can process big endothelin to active endothelin-(1–21) (39, 43). In addition, it has been previously reported that HS loading in Dahl salt-sensitive rats increases blood pressure and renal endothelin expression, which are reversed by an endothelin receptor blocker (17). Furthermore, our data indicate, for the first time, that HS loading increased tissue chymase activity. Thus, these previous and present data might indicate that an endothelin mechanism also contributes to chymase-dependent salt-induced moderate hypertension in mice. This hypothesis needs to be challenged in future study.

In conclusion, the addition of 2% salt to the drinking water of normotensive WT mice caused moderate hypertension and activated the circulating RAS. We demonstrated that the increase of blood pressure with salt loading was dependent on chymase-mediated ANG II formation in the alimentary tract as well as in the skin and skeletal muscle and was independent of ACE. This model may be useful for future studies on salt sensitivity and RAS activity. In addition, a CI suppressed both the elevation of blood pressure and heart rate, along with inhibition of the RAS, indicating a definite involvement of chymase in the salt-sensitive hypertension.

Perspectives
The present study was only performed in male mice, so the effect of salt loading and treatment with the CI need to be investigated in female mice.

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GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES
22. Kefir D, Heinrich R, Kaplan M, Hayek T, Aviram M. Angiotensin II administration to atherosclerotic mice increases macrophage uptake of


