Azilsartan attenuates cardiac damages caused by high salt intake through downregulation of cardiac (pro)renin receptor and its downstream signals in spontaneously hypertensive rats

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Abstract

We examined whether stimulation of the angiotensin II AT1 receptor increases expression of the cardiac (pro)renin receptor ((P)RR) and its downstream signals, and whether blockade of the angiotensin II AT1 receptor by azilsartan decreases expression of the cardiac (P)RR and its signaling in spontaneously hypertensive rats (SHRs) with a high salt intake. Rats received normal-salt (0.9%) chow, high-salt (8.9%) chow, normal-salt chow with 1 mg/day of azilsartan, and high salt chow with 1 mg/day of azilsartan from 6 to 12 weeks of age. Rats with normal-salt chow were administered 100 ng/kg/min of angiotensin II by osmotic minipump from 6 to 12 weeks of age. A high-salt diet and angiotensin II increased significantly the systolic blood pressure and overexpressed cardiac (P)RR, phosphorylated (p)-ERK1/2, p-p38MAPK, p-HSP27, and TGF-ß1 and enhanced cardiac interstitial and perivascular fibrosis, the cardiomyocyte size, interventricular septum (IVS) thickness, and left ventricular (LV) end-diastolic dimension, and decreased LV fractional shortening. Azilsartan decreased the systolic blood pressure, cardiac expressions of the (P)RR, p-ERK1/2, p-p38MAPK, p-HSP27, and TGF-ß1, cardiac interstitial and perivascular fibrosis, the cardiomyocyte size, and LV diastolic dimension and improved LV fractional shortening. In conclusion,

azilsartan attenuates cardiac damages caused by high salt intake through downregulation of cardiac (pro)renin receptor and its downstream signals in SHRs

Keywords: (pro)renin receptor, angiotensin II, angiotensin II AT1 receptor, cardiac damage, hypertension

Introduction

The blood pressure has been reported to be regulated by the renin angiotensin aldosterone system (RAAS) (1). The RAAS has been reported to develop and progress hypertensive heart disease (2). Therefore, the RAAS has been considered as a major pharmacological target of cardiovascular medicine (3, 4).

Prorenin has been considered as a precursor of renin and the kidney is one of the sources of prorenin production (5, 6). Because of the presence of a prosegment covering the enzymatic cleft, prorenin is inactive because prorenin can not bind to angiotensinogen. However, when prorenin binds to the (pro) renin receptor [(P)RR], prorenin becomes active enzymatically because the prosegment is uncovered from the enzymatic cleft and nonproteolytic activation occurs (7).

It has been reported that the binding of prorenin to (P)RR stimulates the (P)RR and triggers intracellular signaling and angiotensin II formed by the conversion of angiotensinogen to angiotensin I by activated prorenin also stimulates the angiotensin II AT1 receptor (8-9). (P)RR has also been reported to be associated with vacuolar-type H^+-ATPase, which maintains intracellular pH (10). It has been reported that the stimulation of (P)RR plays an important role in increasing the left ventricular (LV) mass and deteriorating the LV function in spontaneously hypertensive rats (SHRs) with

excess salt intake (9). We recently reported that high-salt diet markedly accelerated cardiac damage such as LV hypertrophy, LV dysfunction, and LV remodeling through the stimulation of cardiac (P)RR and angiotensin II AT1 receptor by increasing tissue prorenin, renin and angiotensinogen and the activation of signal transductions such as ERK1/2, TGF-β, p38MAPK and HSP27 in SHRs (11). Therefore, the blockade of cardiac (P)RR may attenuate the damage of the heart induced by high salt intake in SHRs.

Handle region decoy peptide (HRP), which has been reported to block (P)RR, has been discovered (12). However, there is controversy over whether HRP blocks (P)RR and its downstream signals. Some studies have suggested that HRP blocks (P)RR and attenuates cardiac fibrosis in stroke-prone SHRs (13) and prevents diabetic nephropathy in diabetic rodents (14). However, other studies have demonstrated that HRP fails to prevent (pro)renin signaling (15) and does not affect hypertensive nephrosclerosis in Goldblatt rats (16). Therefore, a specific (P)RR blocker that is available for clinical use has not yet been established.

Our previous report demonstrated that a high salt intake upregulates the expression of cardiac (P)RR and its downstream signals as well as the expression of cardiac tissue angiotensinogen, which leads to an increase in angiotensin II formation in SHRs,

suggesting that the upregulation of (P)RR and an increase in angiotensin II formation in cardiac tissue occur simultaneously (11). Since (P)RR gene activity was reported to be controlled by intracellular angiotensin II in an in vitro study (17), we hypothesized that cardiac tissue angiotensin II may control the expression of (P)RR in vivo. Therefore, we examined whether the expression of cardiac (P)RR and its downstream signals is enhanced by stimulation of the angiotensin II AT1 receptor and is attenuated by angiotensin II AT1 receptor blocker azilsartan and cardiac damages are attenuated by azilsartan in SHRs with a high salt intake.

Materials and Methods

Experimental animals

Male spontaneously hypertensive rats (SHRs) of 6-week-old, purchased from Chubu Kagaku Sizai Co., Ltd. (Nagoya, Japan), were maintained in animal rooms controlled at temperature of 23±2℃ and humidity of 65±5% with a 12-h light and 12-h dark cycle. All rats were treated in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH publication 85-23, revised in 1996). The protocol of the study was approved by the Committee for Animal Research and Welfare of Gifu University Graduate School of Medicine, Gifu, Japan

(Permit Number 28-10). We measured systolic blood pressure without anesthesia. At the end of the experiment, all rats were sacrificed by an overdose of pentobarbital. To minimize pain and suffering of animals, all the efforts were made.

Protocol 1 (Effect of azilsartan)

Among the many angiotensin receptor blockers (ARBs), azilsartan has been reported to be lipophilic and have a high affinity for tissues (18). Therefore, we used it to block the cardiac tissue angiotensin II AT1 receptor in SHRs.

SHRs of 6 weeks old of age received normal rat chow (0.9% NaCl, CE-2; CLEA Japan, Inc., Tokyo, Japan), high-salt chow (0.9% NaCl CE-2 +8% NaCl: 8.9% NaCl; CLEA Japan, Inc.), normal rat chow + azilsartan (1 mg/30 g normal rat chow, rats normally eat 30 g chow/day; therefore, 1 mg/day (~3 mg/kg/day) of azilsartan), or high-salt chow + azilsartan (1 mg/30 g normal rat chow, rats normally eats 30 g chow/day; therefore, 1 mg/day (~3 mg/kg/day) of azilsartan) for 6 weeks from 6 to 12 weeks old of age (n = 7, respectively). The study groups were SHR + normal-salt (NS group), SHR + high-salt (HS group), SHR + normal-salt + azilsartan (NS+AZ group), and SHR+high-salt + azilsartan (HS+AZ group). As a control, Wistar Kyoto rats

(WKYs) received normal rat chow (0.9% NaCl, CE-2; CLEA Japan, Inc., Tokyo, Japan) from 6 to 12 weeks of age for 6 weeks.

Protocol 2 (Effect of angiotensin II)

SHRs received normal rat chow (0.9% NaCl, CE-2; CLEA Japan, Inc., Tokyo, Japan) (NS group, n=7), high salt rat chow (8.9% NaCl; CLEA Japan, Inc.) (HS group, n=7) or normal rat chow (normal-salt diet, 0.9% NaCl, CE-2; CLEA Japan, Inc., Tokyo, Japan) + 100 ng/kg/min of angiotensin II by subcutaneously implanted osmotic minipump (ALZET, 2006;DURECT, Cupertino, CA, USA) (Ang II group, n=7) for 6 weeks from 6 to 12 weeks old of age. Minipumps were prepared the day before implantation and were incubated in sterile saline at 37 °C overnight. In order to implant osmotic minipump, rats were anesthetized by pentobarbital (30mg/kg, i.c.) to alleviate pain and the minipump was implanted in the subscapular space in either side of the spine. in sterile conditions. Post-operative care was carefully performed aseptically. Following minipump implantation, all animals were allowed ad libitum access to rat chow.

Measurement of blood pressure

We measured the systolic blood pressure from 6 to 12 weeks of age once a week for 6 weeks by the tail-cuff method (BP98-A; Softron Co., Ltd., Tokyo, Japan) in all the rats without anesthesia. The measurement of blood pressure was performed three times on the same animal and the average value was taken as blood pressure.

Echocardiography

At 12 weeks of age, LV fractional shortening (FS), the LV end-diastolic dimension (LVDd), and interventricular septum thickness (IVS) were obtained by echocardiography (Vevo 770; Visualsonics, Toronto, Canada, equipped with a 45-MHz imaging transducer).

Western blot analysis

At 12 weeks of age, the animal was sacrificed and the heart was excised. Western blot analysis was performed by using lysates from the cardiac tissues. By using standard protocols, proteins were transferred to membranes, and then they were probed with antibodies against prorenin, renin (1:100; Santa Cruz Biotechnology, Inc., Dallas, Texas, USA), (pro)renin receptors (1:100; Santa Cruz Biotechnology, Inc.), angiotensinogen (1:200; Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA), angiotensin II AT1

receptor (1:200; Enzo Life Sciences, Inc., Farmingdale, NY, USA), extracellular signal-related kinases (ERK)1/2 (1:100; Cell Signaling Technology, Inc., Danvers, MA, USA), phosphorylated (p)-ERK1/2 (1:100; Cell Signaling Technology, Inc.), transforming growth factor (TGF)-ß1(1:200; Santa Cruz Biotechnology, Inc.), p38 mitogen-activated protein kinase (MAPK) (1:100; Cell Signaling Technology, Inc.), p-p38MAPK (1:100; Cell Signaling Technology, Inc.), heat shock protein (HSP)27 (1:100; Santa Cruz Biotechnology, Inc.), and p-HSP27 (1:100; Santa Cruz Biotechnology, Inc.). The blots were visualized by using chemiluminescence (ECL; GE Healthcare UK Ltd., Amersham Place, Buckinghamshire, England), and the signals were quantified by densitometry. GAPDH (analyzed with an antibody from Cell Signaling Technology, Inc.) was used as the loading control,

Plasma levels of soluble (P)RR

Plasma levels of soluble (P)RR were measured by commercial kit (#27782 soluble (Pro)renin Receptor Assay Kit, Immuno-Biological Laboratores Co., Ltd).

Pathology

At the end of the experiment, the heart was excised, and the left ventricle was weighed and sectioned into two transverse slices parallel to the atrioventricular ring. Slices were fixed in buffered formalin at 10% for 4 h, and then embedded in paraffin, and cut into 4-μm-thick sections by using a microtome. The sections were then stained with Masson-Trichrome and also hematoxylin-eosin, and then observed by light microscopy. Using the sections stained with Masson-Trichrome, the ratio of the myocardial interstitial fibrosis area /total myocardium area was obtained. The diameters of cardiomyocytes were measured by using the sections stained with hematoxylin-eosin. The measurement was performed by 2 persons blinded to treatment.

Statistical analysis

All values are shown as the means ± SEM. Differences among the groups were evaluated by ANOVA combined with Fisher's correction. The value of $p < 0.05$ was considered to be significant.

Additional Materials and Methods are provided in the Online Data Supplement.

Results

Blood pressure

At 12 weeks of age, the systolic blood pressure was significantly higher in the HS group (261 ± 8 mmHg) than in the NS group (183 ± 3 mmHg) and in the WKY group (114 ± 1 mmHg) (Fig. 1-A). The systolic blood pressure was significantly higher in the NS group than in the WKY group.

The systolic blood pressure was significantly decreased in the NS+AZ group (148 ± 4 mmHg) and HS+AZ group than in the HS group (261 ± 8 mmHg). As shown in Fig. 1-B, the high-salt diet significantly and gradually increased the systolic blood pressure from 6 to 12 weeks of age in the SHR groups compared with those fed the normal-salt diet. Azilsartan treatment significantly decreased the systolic blood pressure in the NS+AZ group and HS+AZ group compared with the NS group and HS group from 6 to 12 weeks of age, respectively.

At 12 weeks of age, the systolic blood pressure was significantly higher in the Ang group (274 ± 18 mmHg) and HS group (261 ± 8 mmHg) than in the NS group (183 ± 3 mmHg) (Fig. 1-C). The systolic blood pressure gradually and significantly increased in the HS groups and Ang II group to the same extent, while the NS group showed no change in the systolic blood pressure from 6 to 12 weeks of age (Fig. 1-D). The

systolic blood pressure at each time point was similar between the HS and Ang II groups.

Body weight, heart weight, and heart weight/body weight

As shown in Fig. 2-A, at 12 weeks of age, there was no difference in the body weight between NS and NS+AZ groups, or between HS and HS+AZ groups. The heart weight was significantly higher in the HS group (1.5± 0.04 g) than in the NS group (1.2±0.01 g) (Fig. 2-B). The heart weight was significantly lower in the NS+AZ group than in the NS group, and significantly lower in the HS+AZ group (1.4±0.03 g) than in the HS group (1.5± 0.04 g) (Fig. 2-B). The heart weight/body weight rate was greater significantly in the HS group (0.57± 0.02%) than in the NS group (0.39±0.01%) (Fig. 2-C). The heart weight/body weight rate was significantly lower in the NS+AZ group and HS+AZ group than in the NS group and HS group, respectively (Fig. 2-C).

At 12 weeks of age, there was no difference in the body weight between NS and NS+Ang II groups (Fig. 2-D). The heart weight was significantly greater in the NS+Ang II group than in the NS group (Fig. 2-E). The heart weight/body weight rate was significantly greater in the NS+Ang II group than in the NS group (Fig. 2-F)

Echocardiography

The interventricular septum thickness (IVSth), an indicator of left ventricular hypertrophy, was greater significantly in the HS group (2.3±0.05 mm) than in the NS group (1.9±0.05 mm) (Fig.3-A). The IVSth was significantly thinner in the HS+AZ group than in the HS group (Fig.3-A). The left ventricular end-diastolic dimension (LVDd) was significantly greater in the HS group (6.5±0.11 mm) than in the other groups (Fig.3-B). Fractional shortening (FS) was significantly smaller in the HS group (37.4±0.6) than in the NS group (Fig. 3-C). FS was greater in the HS+AZ group than in the NS group (Fig.3-F).

The IVSth was significantly thicker in the NS+Ang II group than in the NS group (Fig.3-D). LVDd was significantly greater in the NS+Ang II group than in the NS group (Fig.3-E).

Expressions of cardiac (P)RR and its downstream signals

Western blot analysis showed that high salt intake significantly enhanced the expressions of cardiac prorenin in the SHRs (Fig. 4-A). Increased cardiac tissue expression of prorenin was attenuated by azilsartan (Fig. 4-A). High salt intake significantly enhanced the cardiac expressions of (P)RR in the SHRs (Fig. 4-B), and

this increase was attenuated by azilsartan (Fig. 4-B). (P)RR's downstream ERK1/2 was not different among the groups (Fig. 4-C), but p-ERK1/2 signal was upregulated by high salt intake and this upregulation was attenuated by azilsartan (Fig. 4-D). The upregulated expressions of HSP27 and p-HSP27 in the HS group were attenuated by azilsartan (Fig.4-E, F). The upregulated expressions of p-p38MAPK and TGF-ß1 in the HS group were attenuated by azilsartan (Fig. 4-G, H).

There was no difference in cardiac expression of prorenin between NS and NS+Ang II groups (Fig. 5-A). Cardiac expression of (P)RR was significantly greater in the NS+Ang II group than in the NS group (Fig.5-B). There was no difference in cardiac expression of ERK1/2 between NS and NS+Ang II groups (Fig. 5-C). Cardiac expressions of p-ERK1/2, HSP and p-HSP were significantly higher in the NS+Ang II group than in the NS group (Fig.5-D, E, F). There was no difference in cardiac expression of p38MAPK between NS and NS+Ang II groups (Fig. 5-G). Cardiac expressions of TGF-ß1 were significantly higher in the NS+Ang II group than in the NS group (Fig.5-I)

Plasma levels of soluble (P)RR

Plasma level of soluble (pro) renin receptor was significantly elevated in the HS group ($p<0.05$) but not in the NS+AZ group or HS+AZ group than in the NS group (Fig. 5-K).

Pathology

The representative short axis sections of the left ventricle stained with Masson-Trichrome was shown in Fig. 6-A. The high salt diet fascilitated the development of myocardial interstitial fibrosis in SHRs (Figs. 6-B). Angiotensin II also fascilitated the development of myocardial interstitial fibrosis in SHRs (Fig. 6-B). Azilsartan attenuated the myocardial interstitial fibrosis in the HS group (Fig.6-B). Perivascular fibrosis was more marked in the HS and Ang II groups than in the NS group (Fig.6-C). Azilsartan reduced perivascular fibrosis in the HS group (Fig. 6-C). The ratio of the myocardial interstitial fibrosis area/myocardium was significantly attenuated in the NS+AZ group and HS+AZ group compared with those in the NS and HS groups, respectively (Fig. 6-E). The diameter of cardiomyocytes was greater in the HS group than in the NS group (Fig.6-D, F). Cardiomyocyte diameter significantly decreased in the HS+AZ group compared with the HS group (Fig. 6-D, F). Angiotensin II enhanced the rate of myocardial interstitial fibrosis/myocardium

compared with those of the NS group (Fig. 6-B, G). The diameter of cardiomyocytes was significantly larger in the Ang II group than in the NS group (Fig 6-D, H).

Discussion

We observed in the present study that a high salt intake significantly more increased the systolic blood pressure in the SHR group compared with those fed a normal-salt diet (Fig. 1-A, B). This result is consistent with studies previously reported including our report that the blood pressure is increased by a high-salt diet (9, 11, 19, 20). It has been reported that the blood pressure is increased by a high salt intake because of increased volume load and calcium entry into vascular smooth muscle cells and constriction via Na^{+}/Ca^{2+} exchanger NCX1 (21). Azilsartan significantly decreased the systolic blood pressure in the NS and HS groups (Fig. 1-A, B). Angiotensin II increased the systolic blood pressure to the same extent as that in the HS group (Fig. 1-C, D).

The heart weight was the greatest in the HS group and the heart weight/ body weight ratio was the greatest in the HS group among the groups at 12 weeks of age (Fig.2-B, C). The heart weight and heart weight/body weight ratio in the NS and HS groups were significantly decreased by treatment with azilsartan (Fig.2-B, C). Angiotensin II increased the heart weight and heart weight/body weight ratio in the NS

group (Fig.2-E, F). The IVSth, a marker of left ventricular hypertrophy, assessed by echocardiography was greater in the HS group than in the other groups, as shown in Fig. 3-A. This may have been mainly due to a higher systolic blood pressure in SHRs caused by a high salt intake. The activation of p38MAPK and HSP27 may also have contributed to the increase in the IVSth in the HS group (Fig.3-A). However, azilsartan significantly decreased the IVSth in the HS+AZ group compared with the HS group (Fig. 3-A). It is consistent with the change in signal transduction that the expressions of p-p38MAPK and p-HSP27, which accelerate cardiomyocyte hypertrophy, were significantly decreased in the HS+AZ group compared with those in the HS group (Fig.4-F, G), suggesting that azilsartan reduced the LV wall thickness by decreasing the activation of p38MAPK and HSP27 in addition to a decrease in the systolic blood pressure. Angiotensin II significantly increased the IVSth (Fig. 3-D). The left ventricular end-diastolic dimension (LVDd), an indicator of heart failure, was greater in the HS group than in the other groups (Fig. 3-B). This may have been due to a higher systolic blood pressure at least in part and activation of TGF-ß1, which might have facilitated cardiac interstitial fibrosis. However, azilsartan significantly decreased the LVDd in the HS+AZ group compared with that in the HS group (Fig. 3-B). Angiotensin II significantly increased the LVDd (Fig. 3-E). Fractional shortening (FS), an indicator

of the cardiac function, was significantly smaller in the HS group compared with the other groups. Deteriorated FS in the HS group may have been caused by an increase in the systolic blood pressure, an afterload to the left ventricle, as well as cardiac interstitial fibrosis caused by an upregulation of TGF-ß1 (Fig.4-H). Angiotensin II significantly decreased the FS (Fig. 3-F).

We previously reported that the expressions of prorenin, renin, (P)RR, angiotensinogen and angiotensin II AT1 receptor in the left ventricle were significantly augmented by a high salt intake in the SHR group in spite of PRA being low (11). It has been reported that the binding of prorenin and renin to (P)RR triggers intracellular signaling and activates extracellular signal-related kinases (ERK)1/2, and then leads to the upregulation of TGF-ß1, and then induces fibrosis (22, 23). The activation of (P)RR has also been reported to contributes to the cardiac fibrosis development in genetic hypertension (24). Furthermore, it has been reported that stimulation of (P)RR triggers the activation of p38 MAPK, and then leads to the upregulation of HSP27, which is reported to enhance the DNA synthesis and causes cardiomyocyte hypertrophy (25, 26). We previously reported that a high salt intake significantly increases the expressions of (P)RR, angiotensinogen, and angiotensin II AT1 receptor and activates ERK1/2, p38 MAPK, HSP27, and TGF-ß1 in the left ventricle tissues, leading to cardiac interstitial

fibrosis, perivascular fibrosis, and cardiac hypertrophy in SHRs (8). In the present study, the high salt intake again increased the expressions of prorenin, (P)RR and p-ERK1/2, p-p38 MAPK, p-HSP27, and TGF-ß1 in the left ventricle in the SHRs (Fig. 4-A, B, D, F, G, H). However, azilsartan significantly decreased the expressions of prorenin, (P)RR, p-ERK1/2, p-p38 MAPK, p-HSP27, and TGF-ß1 caused by the high salt intake (Fig. 4-A, B, D, F, G, H). In the present study, treatment with angiotensin II significantly increased the expressions of (P)RR, p-ERK1/2, p-HSP27, p-p38MAPK, and TGF-ß1 in left ventricle tissue in the NS+Ang II group compared with those in the NS group (Fig.5-B, D, F, H, I). Consistent with the behavior of expression of cardiac tissue (P)RR, plasma level of soluble (P)RR was significantly elevated in the HS group ($p<0.05$) but not in the NS+AZ group or HS+AZ group as compared to that in the NS group (Fig. 5-K).

Pathologically, cardiac interstitial and perivascular fibrosis, and cardiomyocyte hypertrophy were fascilitated in the HS group (Fig. 6-B, C, D, E, F). These suggest that the combination of a high salt intake and hypertension accelerates cardiac interstitial and perivascular fibrosis, and cardiomyocyte hypertrophy in SHRs. Azilsartan significantly decreased the cardiac interstitial fibrosis area in the NS and HS groups (Fig. 6-E). Azilsartan significantly decreased the cardiomyocyte size (Fig. 6-F). Conversely,

angiotensin II significantly increased the cardiac fibrosis area and cardiomyocyte size (Fig. 6-G, H).

In conclusion, findings in the present study suggest that angiotensin II regulates cardiac (P)RR and that ARB azilsartan prevents cardiac damage through attenuating the upregulation of cardiac (P)RR and its downstream signals.

Study limitation

The present study may lack the direct data supporting the critical role of (P)RR in the cardiac damage in SHRs with high salt diet because azilsartan is not a direct blocker of (P)RR. In an attempt to clarify the direct role of (P)RR in cardiac damage in SHRs with high salt intake, we investigated the effects of handle region peptide (HRP), which is reported to block (P)RR (27). As a result, HRP did not affect hypertension, expression of cardiac (P)RR, its downstream signals, cardiac pathology or cardiac function (Online Figures I, II and III). The reason why HRP did not affect the above mentioned parameters is unclear. However, there are still controversies whether HRP blocks (P)RR and its downstream signals (28, 29, 30, 31). Therefore, one of the explanations for different effects of HRP may be caused by the differences in the pathophysiological status in different disease models of rats. Another explanation may be due to the doses of HRP administered. However, this seems to be unlikely because

the dose of HRP used in the present study (0.1 mg/kg/day for 6 weeks) was much higher than those of other previous experiments (28, 29, 30, 31).

Furthermore, we compared the effect of antihypertensive diuretics trichlormethiazide, which may decrease blood pressure by decreasing the volume overload on the heart, with that of azilsartan. As a result, treatment with trichlormethiazide decreased the systolic blood pressure to the same extent as that in the azilsartan (Online Figure IV) but did not affect the expression of cardiac (P)RR (Online Figure V). These results may exclude the possibility that the results of azilsartan were just mediated by changes in pressure or volume overload on the heart in a non-specific manner to (P)RR.

In addition, we compared the effect of losartan, a hydrophilic ARB, with that of azilsartan, a lipophilic ARB. As a result, treatment with losartan decreased the systolic blood pressure to the same extent as that in the azilsartan (Online Figure VI) but did not affect the expression of cardiac (P)RR (Online Figure VII). This suggests that the effect of azilsartan to decrease the expression of cardiac (P)RR is not a class effect of ARBs but a peculiar effect of azilsartan.

Clinical Implication

Since the use of azilsartan is clinically available in patients with hypertension at present, azilsartan may be a realistic indirect blocker of (P)RR to prevent (P)RR signaling as well as a direct blocker of angiotensin II AT1 receptor.

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Figure Legends

Figure 1　Changes in the systolic blood pressure

A: Systolic blood pressure at 12 weeks of age in each group, WKY group = WKY + normal-salt (n = 7), NS group = SHR + normal-salt (n = 7), HS group= SHR + high-salt (n = 7), NS+AZ group=SHR+normal-salt+azilsartan, HS+AZ group = SHR+high-salt+azilsartan,

*: $p < 0.05$, **: $p < 0.01$

B: Time-course changes in systolic blood pressure in response to a high salt intake and azilsartan (AZ)

WKY group＝WKY + normal-salt (n = 7), NS group = SHR + normal-salt (n = 7), HS group= SHR + high-salt (n = 7), NS+AZ group=SHR+normal-salt+azilsartan, HS+AZ group=SHR+high-salt+azilsartan (n=7),

C: Effect of angiotensin II on systolic blood pressure in the SHRs with normal-salt at 12 weeks of age. Systolic blood pressures in the NS+Ang II was similar to that in the HS group.

NS group = SHR + normal-salt (n=7), HS group= SHR + high-salt (n=7), NS+Ang II group = SHR+normal-salt+angiotensin II (n=7), *: $p < 0.05$

D: Time-course changes in systolic blood pressure in response to angiotensin II in the SHR groups. Changes in systolic blood pressure in the NS+Ang II group were similar to those in the HS group.

Figure 2 Heart weight, lung weight, body weight, heart weight/body weight rate and lung weight/ body weight rate

A: Body weight at 12 weeks of age in each group

B: Heart weight at 12 weeks of age in each group

C: Heart weight/ body weight ratio at 12 weeks of age in each group

D: Effect of angiotensin II on body weight at 12 weeks of age

E: Effect of angiotensin II on heart weight at 12 weeks of age

F: Effect of angiotensin II on heart weight/body weight rate at 12 weeks of age

NS group = SHR+normal-salt group (n = 7), HS group = SHR + high-salt (n = 7), NS+AZ group=SHR+ normal-salt + azilsartan (n = 7), HS+AZ group = SHR + high-salt +azilsartan (n = 7), NS+Ang II group=SHR+normal-salt+angiotensin II, *: $p < 0.05$, **; $p < 0.01$

Figure 3 Echocardiographic findings

A: Interventricular septum thickness (ICSth) at 12 weeks of age

B: Left ventricular diastolic dimension (LVDd) at 12 weeks of age

C: Left ventricular fractional shortening (FS) at 12 weeks of age

D: Effect of angiotensin II and HRP on IVSth at 12 weeks of age

E: Effect of angiotensin II and HRP on LVDd at 12 weeks of age

F: Effect of angiotensin II and HRP on FS at 12 weeks of age

NS group = SHR+normal-salt group (n = 7), HS group = SHR + high-salt (n = 7), NS+AZ group=SHR+ normal-salt + azilsartan (n = 7), HS+AZ group = SHR + high-salt +azilsartan (n = 7), NS+Ang II group=SHR+normal-salt+angiotensin II, *: $p < 0.05$, **: $p < 0.01$

Figure 4 Expression of cardiac tissue prorenin and (pro)renin receptor and its downstream signals

A: expression of prorenin, B: expression of cardiac (pro)renin receptor, C: expression of ERK1/2, D: expression of p-ERK1/2, E: expression of HSP27, F: expression of p-HSP27, G: expression of p-p38MAPK, H: expression of TGF-ß

NS group = SHR+normal-salt group (n = 7), HS group = SHR + high-salt (n = 7), NS+AZ group=SHR+ normal-salt + azilsartan (n = 7), HS+AZ group = SHR + high-salt +azilsartan (n = 7), *: $p < 0.05$

Figure 5 Effect of angiotensin II on expression of prorenin, (pro)renin receptor and its downstream signals, and plasma levels of soluble (P)RR

A: expressions of prorenin, B: expression of cardiac (pro) renin receptor=(P)RR, C: expression of ERK1/2, D: expression of p-ERK1/2, E: expression of HSP27, F: expression of p-HSP27, G: expression of p38MAPK, H: expression of p-p38MAPK, I: expression of TGF-ß1, J: GAPDH, NS group = SHR+normal-salt group (n = 7), NS+Ang II group = SHR+normal-salt+angiotensin II (n=7), *: $p < 0.05$

K: Plasma levels of soluble (P)RR, NS group = SHR+normal-salt group (n = 4), HS group=SHR+high-salt group (n=4), NS+AZ group=SHR+ normal-salt + azilsartan (n = 8), HS+AZ group = SHR + high-salt +azilsartan (n = 8), *: $p < 0.05$

Figure 6 Masson-trichrome staining and hematoxylin-eosin staining of left ventricle

A: Representative short-axis sections of cardiac ventricle stained with Masson-trichrome Scale bar: 1 mm.

B: Representative short-axis images of the myocardium and interstitial fibrosis stained with Masson-trichrome. Scale bar: 50 μm.

C: Representative short-axis images of the myocardium and perivascular fibrosis stained with Masson-Trichrome. Scale bar: 50 μm.

D: Representative short-axis images of the myocardium stained with hematoxylin-eosin Scale bar: 50 μm

E: Rate of myocardial interstitial fibrosis area/myocardium at 12 weeks of age in the WKY, NS, NS+AZI, HS and HS+AZI groups

F: Short-axis diameter of cardiomyocytes at 12 weeks of age in the WKY, NS, NS+AZI, HS and HS+AZI groups

G: Rate of myocardial interstitial fibrosis area/myocardium at 12 weeks of age in the NS, NS+Ang II and HS groups

H: Short-axis diameter of cardiomyocytes at 12 weeks of age in the NS, NS+Ang II and HS groups

NS group = SHR+normal-salt group (n = 7), HS group = SHR + high-salt (n = 7), NS+AZI group=SHR+ normal-salt + azilsartan (n = 7), HS+AZI group = SHR + high-salt +azilsartan (n = 7), NS+Ang II group=SHR+normal-salt+angiotensin II, *: $p < 0.05$, **: $p < 0.01$