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ARTICLE

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

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Abstract

We examined whether the stimulation of the angiotensin II AT1 receptor increases the expression of the cardiac (pro)renin receptor ((P)RR) and its downstream signals and whether the blockade of the angiotensin II AT1 receptor by azilsartan decreases the 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 significantly increased the systolic blood pressure; overexpressed cardiac (P)RR, phosphorylated (p)-ERK1/2, p-p38MAPK, p-HSP27, and TGF-ß1; enhanced cardiac interstitial and perivascular fibrosis, cardiomyocyte size, interventricular septum (IVS) thickness, and left ventricular (LV) end-diastolic dimension; and decreased LV fractional shortening. Azilsartan decreased systolic blood pressure, cardiac expressions of (P)RR, p-ERK1/2, p-p38MAPK, p-HSP27, and TGF-ß1, cardiac interstitial and perivascular fibrosis, cardiomyocyte size, and LV diastolic dimension, and improved LV fractional shortening. In conclusion, azilsartan attenuates cardiac damage caused by high salt intake through the downregulation of the cardiac (pro) renin receptor and its downstream signals in SHRs.

Introduction

Blood pressure has been reported to be regulated by the renin–angiotensin aldosterone system (RAAS) [1]. The RAAS is involved in the development and progression of 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 cannot 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 a 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,

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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 and 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 the stimulation of the angiotensin II AT1 receptor and attenuated by angiotensin II AT1 receptor blocker azilsartan, and whether cardiac damage is attenuated by azilsartan in SHRs with high salt intake.

Materials and methods

Experimental animals

SHRs, purchased from Chubu Kagaku Sizai Co., Ltd. (Nagoya, Japan), were maintained in animal rooms controlled at a temperature of 23 ± 2 °C 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 killed by an overdose of pentobarbital. All efforts were made to minimize the pain and suffering of animals.

Protocol 1 (effect of azilsartan)

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

Six-week-old SHRs 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 eat 30 g chow/day; therefore, 1 mg/day (~3 mg/kg/day) of azilsartan) for 6 weeks for rats that are 6–12 weeks old ($n = 7$, 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 of age. Minipumps were prepared the day before implantation and incubated in sterile saline at 37 °C overnight. To implant an osmotic minipump, rats were anesthetized by pentobarbital (30 mg/kg, i.c.) to alleviate pain, and the minipump was implanted in the subscapular space on either side of the spine in sterile conditions. Postoperative 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 in SHRs 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 rats without anesthesia. The measurement of blood pressure was performed three times on the same animal, and the average value was taken as the 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 animals were killed, and the hearts were excised. Western blot analysis was performed by using lysates from cardiac tissues. Using standard protocols, proteins were transferred to membranes, and 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 using chemiluminescence (ECL; GE Healthcare UK Ltd., Amersham Place, Buckinghamshire, England), and the signals were quantified by densitometry. Glyceraldehyde-3-phosphatedehydrogenase (GAPDH) (analyzed with antibodies 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 a commercial kit (#27782 soluble (Pro)renin Receptor Assay Kit, Immuno-Biological Laboratories Co., Ltd).

Pathology

At the end of the experiment, the hearts were excised, and the left ventricles were weighed and sectioned into two transverse slices parallel to the atrioventricular ring. Slices were fixed in buffered formalin at 10% for 4 h, embedded in paraffin, and cut into 4-µm-thick sections using a microtome. The sections were then stained with Masson trichrome and 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 using the sections stained with hematoxylin–eosin. The measurement was performed by two persons blinded to treatment.

Statistical analysis

All values are shown as the mean ± 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 that in the NS group (183 ± 3 mmHg) and WKY group (114 ± 1 mmHg) (Fig. 1a). The systolic blood pressure was significantly higher in the NS group than that in the WKY group.

The systolic blood pressure was significantly decreased in the NS + AZ group (148 ± 4 mmHg) and HS + AZ group than that in the HS group (261 ± 8 mmHg). As shown in Fig. 1b, 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 with a 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 that in the NS group (183 ± 3 mmHg) (Fig. 1c). 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. 1d). 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. 2a, at 12 weeks of age, there was no difference in the body weight between NS and NS + AZ groups or between the HS and HS + AZ groups. The heart weight was significantly higher in the HS group (1.5 ± 0.04 g) than that in the NS group (1.2 ± 0.01 g) (Fig. 2b). The heart weight was significantly lower in the NS + AZ group

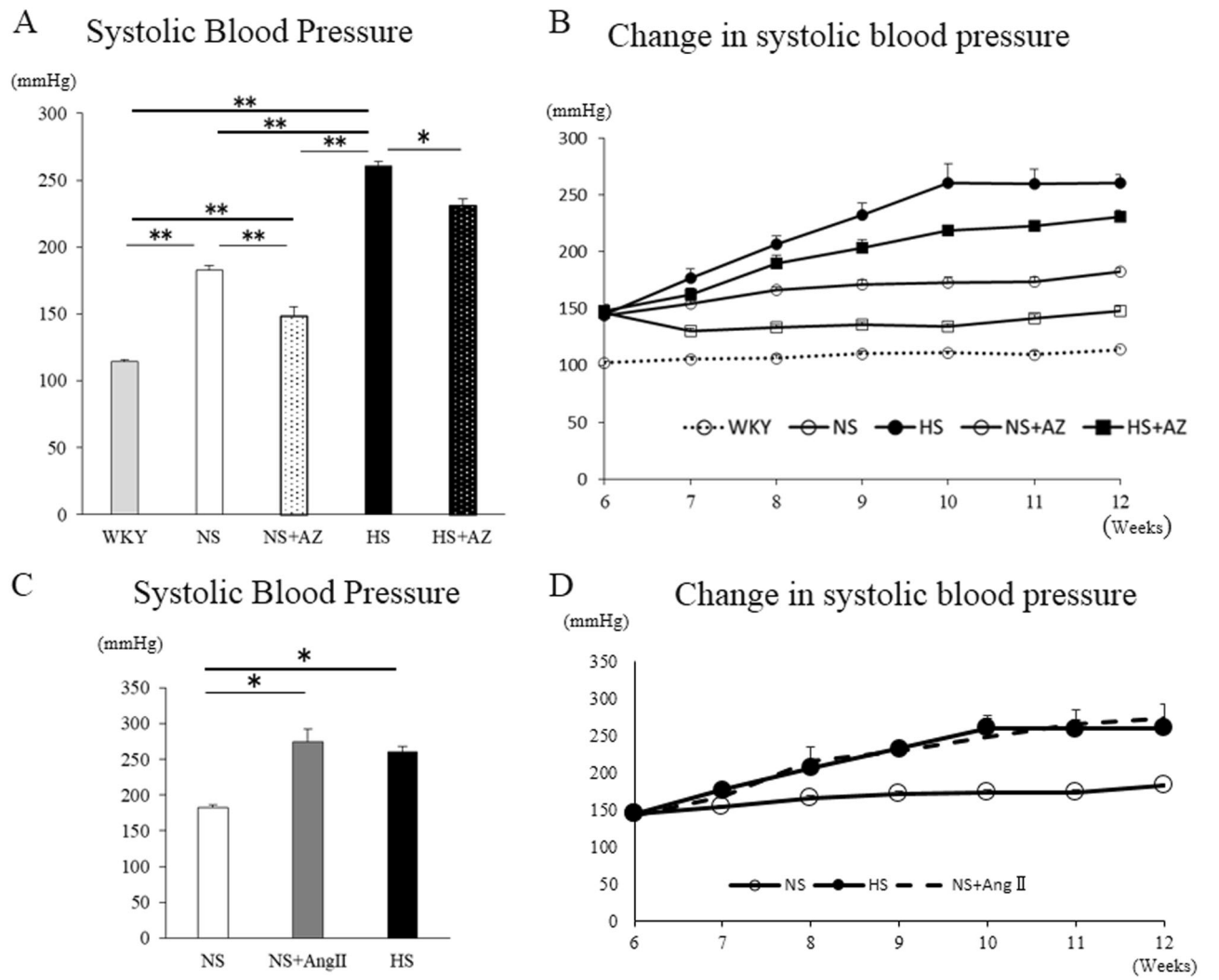

Fig. 1 Changes in the systolic blood pressure. **a** Systolic blood pressure at 12 weeks of age in each group: WKY group = WKY + normal salt ($n = 7$); NS group = SHR + normal-salt ($n = 7$); HS group = SHR + high-salt ($n = 7$); NS + AZ group = SHR + normal-salt + azilsartan; HS + AZ group = SHR + high-salt + 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

than that in the NS group and significantly lower in the HS + AZ group (1.4 ± 0.03 g) than that in the HS group (1.5 ± 0.04 g) (Fig. 2b). The heart weight/body weight rate was significantly greater in the HS group (0.57 ± 0.02%) than that in the NS group (0.39 ± 0.01%) (Fig. 2c). The heart weight/body weight rate was significantly lower in the NS + AZ group and HS + AZ group than that in the NS group and HS group, respectively (Fig. 2c).

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

Echocardiography

The interventricular septum thickness (IVSth), an indicator of left ventricular hypertrophy, was significantly greater in the HS group (2.3 ± 0.05 mm) than that in the NS group (1.9 ± 0.05 mm) (Fig. 3a). The IVSth was significantly thinner in the HS + AZ group than that in the HS group (Fig. 3a). The left ventricular end-diastolic dimension (LVDd) was significantly greater in the HS group (6.5 ± 0.11 mm) than that in the other

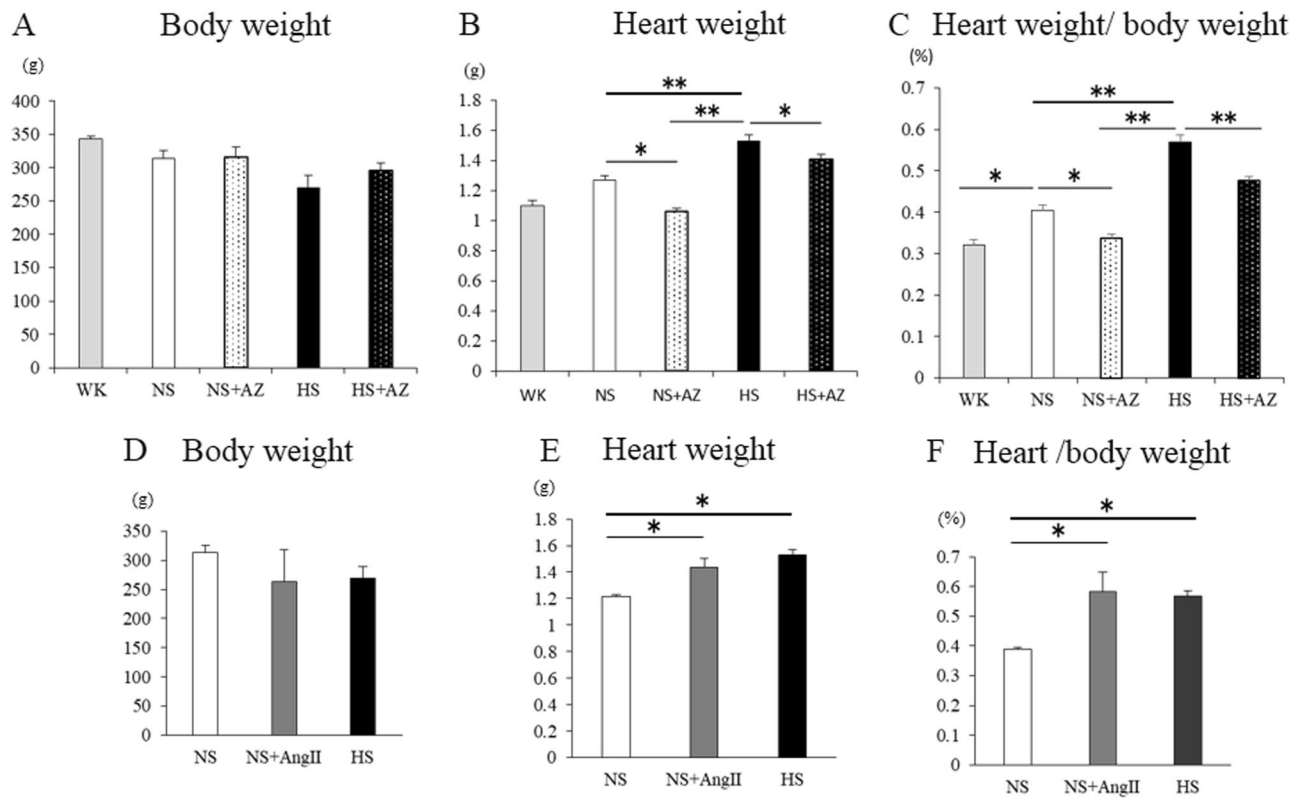

Fig. 2 Heart weight, lung weight, body weight, heart weight/body weight rate, and lung weight/body weight rate. **a** Body weight at 12 weeks of age in each group. **b** Heart weight at 12 weeks of age in each group. **c** Heart weight/body weight ratio at 12 weeks of age in each group. **d** Effect 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; $^{*}p < 0.05$, $^{**}p < 0.01$

groups (Fig. 3b). Fractional shortening (FS) was significantly smaller in the HS group (37.4 ± 0.6) than that in the NS group (Fig. 3c). FS was greater in the HS + AZ group than that in the NS group (Fig. 3f).

The IVSth was significantly thicker in the NS + Ang II group than that in the NS group (Fig. 3d). LVDd was significantly greater in the NS + Ang II group than that in the NS group (Fig. 3e).

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. 4a). The increased cardiac tissue expression of prorenin was attenuated by azilsartan (Fig. 4a). High salt intake significantly enhanced the cardiac expressions of (P)RR in the SHRs (Fig. 4b), and this increase was attenuated by azilsartan (Fig. 4b). (P)RR's downstream ERK1/2 was not different among the groups (Fig. 4c), but the p-ERK1/2 signal was upregulated by high salt intake, and this upregulation was attenuated by azilsartan (Fig. 4d). The upregulated expressions of HSP27 and p-HSP27 in the HS group were attenuated by azilsartan (Fig. 4e, f). The upregulated expressions of p-p38MAPK and TGF-ß1 in the HS group were attenuated by azilsartan (Fig. 4g, h).

There was no difference in the cardiac expression of prorenin between the NS and NS + Ang II groups (Fig. 5a). The cardiac expression of (P)RR was significantly greater in the NS + Ang II group than that in the NS group (Fig. 5b). There was no difference in the cardiac expression of ERK1/2 between the NS and NS + Ang II groups (Fig. 5c). Cardiac expressions of p-ERK1/2, HSP, and p-HSP were significantly higher in the NS + Ang II group than those in the NS group (Fig. 5d, e, f). There was no difference in the cardiac expression of p38MAPK between the NS and NS + Ang II groups (Fig. 5g). Cardiac expressions of TGF-ß1 were significantly higher in the NS + Ang II group than those in the NS group (Fig. 5i, j).

Plasma levels of soluble (P)RR

The plasma level of soluble (pro) renin receptor was significantly elevated in the HS group ($p < 0.05$) but not in the

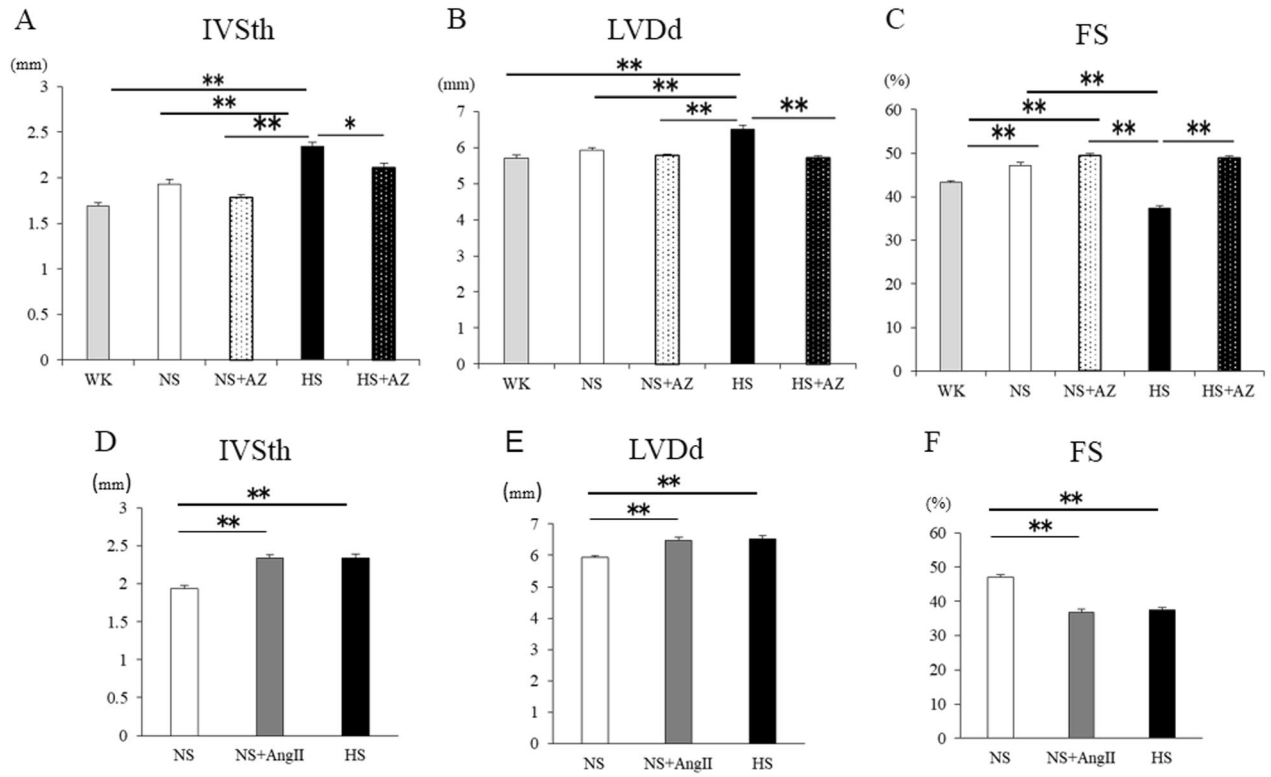

Fig. 3 Echocardiographic findings. **a** Interventricular septum thickness (ICSth) at 12 weeks of age. **b** Left ventricular diastolic dimension (LVDd) at 12 weeks of age. **c** Left ventricular fractional shortening (FS) at 12 weeks of age. **d** Effect of angiotensin II and HRP on IVSth at 12 weeks 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; $^{*}p < 0.05$, $^{**}p < 0.01$

NS + AZ group or HS + AZ group compared to the NS group (Fig. 5k).

Pathology

The representative short-axis sections of the left ventricle stained with Masson trichrome are shown in Fig. 6a. The high-salt diet facilitated the development of myocardial interstitial fibrosis in SHRs (Fig. 6b). Angiotensin II also facilitated the development of myocardial interstitial fibrosis in SHRs (Fig. 6b). Azilsartan attenuated the myocardial interstitial fibrosis in the HS group (Fig. 6b). Perivascular fibrosis was more marked in the HS and Ang II groups than that in the NS group (Fig. 6c). Azilsartan reduced perivascular fibrosis in the HS group (Fig. 6c). 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. 6e). The diameter of cardiomyocytes was greater in the HS group than that in the NS group (Fig. 6d, f). The cardiomyocyte diameter significantly decreased in the HS + AZ group compared with that in the HS group (Fig. 6d, f). Angiotensin II enhanced the rate of myocardial interstitial fibrosis/myocardium compared with that of the NS group (Fig. 6b, g). The diameter of cardiomyocytes was significantly larger in the Ang II group than that in the NS group (Fig. 6d, h).

Discussion

In the present study, we observed that a high salt intake significantly increased the systolic blood pressure in the SHR group compared with those fed with a normal-salt diet (Fig. 1a, b). This result is consistent with previously reported studies, 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

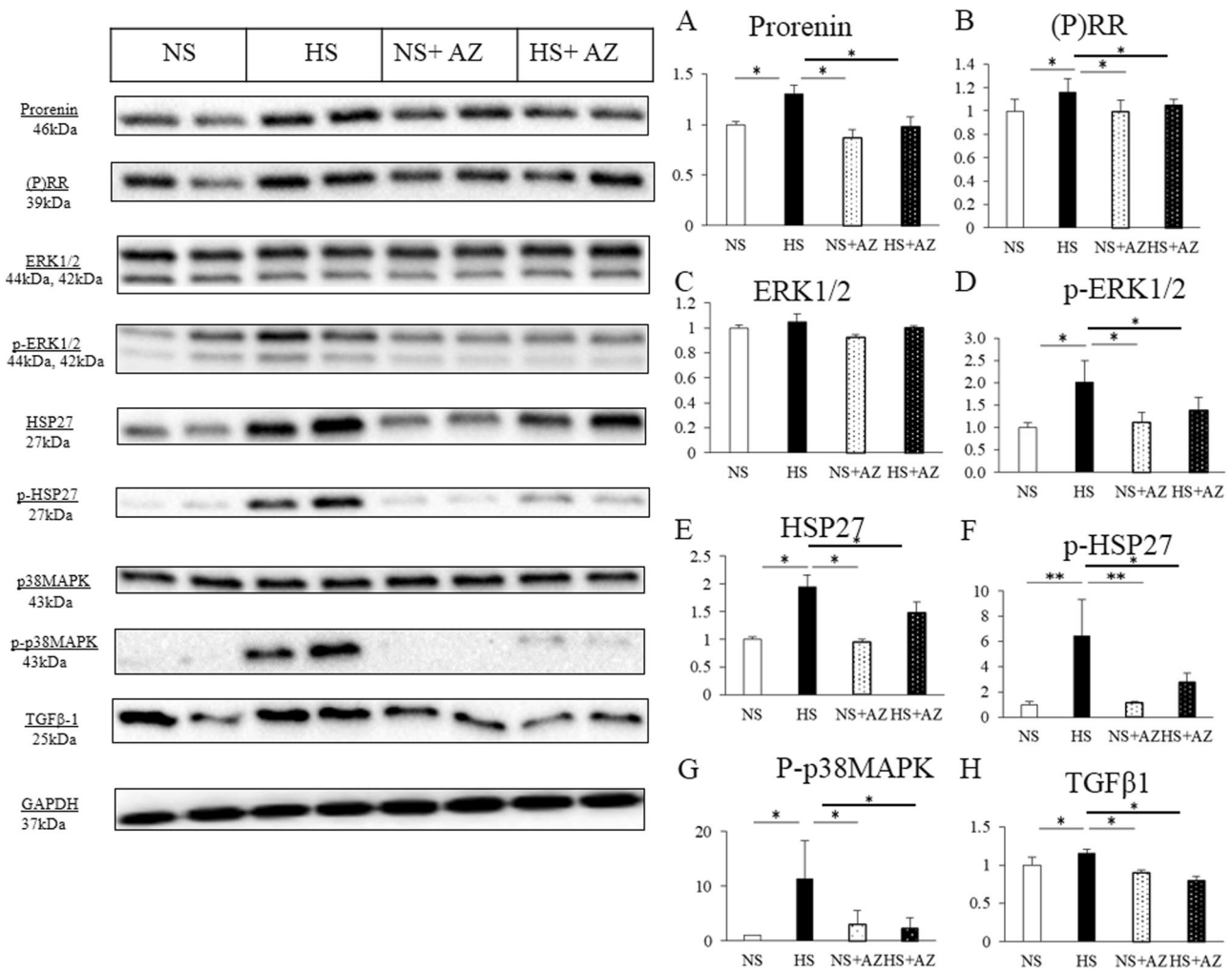

Fig. 4 Expression of cardiac tissue prorenin and (pro)renin receptor and its downstream signals. **a** Expression of prorenin; **b** expression of cardiac (pro)renin receptor; **c** expression of ERK1/2; **d** expression of p-ERK1/2; **e** expression of HSP27; **f** expression of p-HSP27; **g** expression 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 ($n = 7$); $^*p < 0.05$

decreased the systolic blood pressure in the NS and HS groups (Fig. 1a, b). Angiotensin II increased the systolic blood pressure to the same extent as that in the HS group (Fig. 1c, 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. 2b, c). The heart weight and heart weight/body weight ratio in the NS and HS groups were significantly decreased by treatment with azilsartan (Fig. 2b, c). Angiotensin II increased the heart weight and heart weight/body weight ratio in the NS group (Fig. 2e, f). The IVSth, a marker of left ventricular hypertrophy, assessed by echocardiography was greater in the HS group than that in the other groups, as shown in Fig. 3a. 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. 3a). However, azilsartan significantly decreased the IVSth in the HS + AZ group compared with that in the HS group (Fig. 3a). This is consistent with the change in signal transduction where the expressions of p-p38MAPK and p-HSP27, which accelerate cardiomyocyte hypertrophy, significantly decreased in the HS + AZ group compared with those in the HS group (Fig. 4f, 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. 3d). The left ventricular end-diastolic dimension (LVDd), an indicator of heart failure, was greater in the HS group than in the other groups (Fig. 3b). This may have been due to a higher systolic blood pressure at least in part and the activation of TGF-ß1, which might have facilitated cardiac interstitial fibrosis. However,

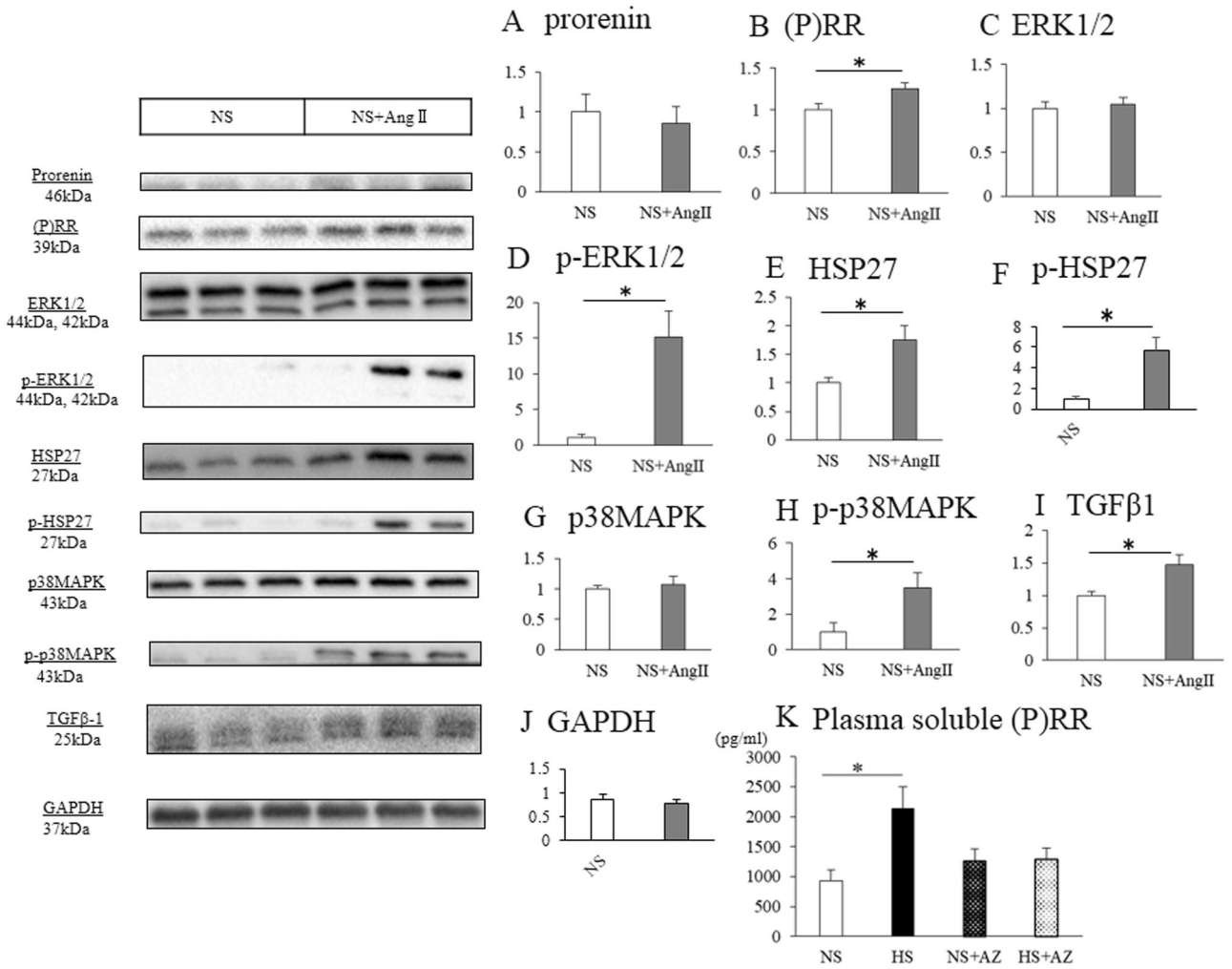

Fig. 5 Effect of angiotensin II on the expression of prorenin, (pro)renin receptor and its downstream signals, and plasma levels of soluble (P)RR. **a** Expressions of prorenin; **b** expression of cardiac (pro) renin receptor = (P)RR; **c** expression of ERK1/2; **d** expression of p-ERK1/2; **e** expression of HSP27; **f** expression of p-HSP27; **g** expression of p38MAPK; **h** expression of p-p38MAPK; **i** expression of TGF-β1; **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 ($n = 8$); $^*p < 0.05$

azilsartan significantly decreased the LVDd in the HS + AZ group compared with that in the HS group (Fig. 3b). Angiotensin II significantly increased the LVDd (Fig. 3e). 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, afterload to the left ventricle, and cardiac interstitial fibrosis caused by the upregulation of TGF-ß1 (Fig. 4h). Angiotensin II significantly decreased the FS (Fig. 3f).

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, leads to the upregulation of TGF-β1, and then induces fibrosis [22, 23]. The activation of (P)RR has also been reported to contribute to the development of cardiac fibrosis in genetic hypertension [24]. Furthermore, it has been reported that the 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 cause cardiomyocyte hypertrophy [8, 25]. 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

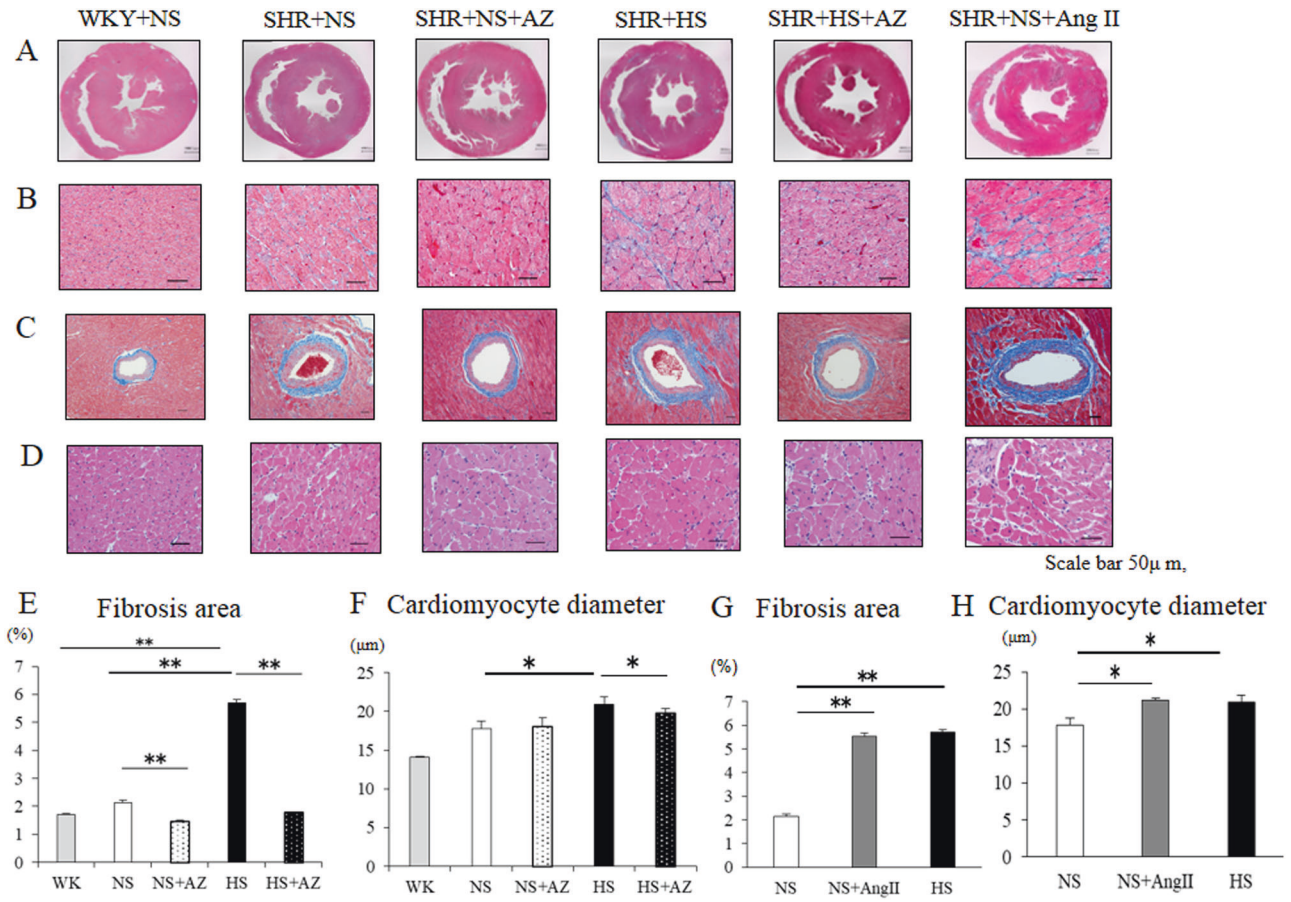

Fig. 6 Masson-trichrome staining and hematoxylin–eosin staining of the left ventricle. **a** Representative short-axis sections of the cardiac ventricle stained with Masson trichrome. Scale bar: 1 mm. **b** Representative short-axis images of the myocardium and interstitial fibrosis stained with Masson 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; $^{*}p < 0.05$, $^{**}p < 0.01$

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-β1 in the left ventricle in the SHRs (Fig. 4a, 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-β1 caused by the high salt intake (Fig. 4a, 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-β1 in left ventricle tissue in the NS + Ang II group compared with those in the NS group (Fig. 5b, d, f, h, i, j). Consistent with the behavior of the expression of cardiac tissue (P)RR, the 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 compared to that in the NS group (Fig. 5k).

Pathologically, cardiac interstitial and perivascular fibrosis and cardiomyocyte hypertrophy were facilitated in the HS group (Fig. 6b–f). This suggests 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. 6e). Azilsartan significantly decreased the cardiomyocyte size (Fig. 6f). Conversely, angiotensin II significantly increased the cardiac fibrosis area and cardiomyocyte size (Fig. 6g, h).

In conclusion, the findings of the present study suggest that angiotensin II regulates cardiac (P)RR, and ARB

azilsartan prevents cardiac damage by attenuating the upregulation of cardiac (P)RR and its downstream signals.

Study limitations

The present study lacks direct data supporting the critical role of (P)RR in the cardiac damage in SHRs with a 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 the handle region peptide (HRP), which is reported to block (P)RR [26]. As a result, HRP did not affect hypertension, the expression of cardiac (P)RR, its downstream signals, cardiac pathology, or cardiac function (Supplementary Fig. 1, 2, and 3). The reason why HRP did not affect the above-mentioned parameters is unclear. However, there is still controversy about whether HRP blocks (P)RR and its downstream signals [13–16]. Therefore, one of the explanations for different effects of HRP may be caused by the differences in the pathophysiological status in the 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 [13–16].

Furthermore, we compared the effect of the antihypertensive diuretic 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 (Supplementary Fig. 4) but did not affect the expression of cardiac (P)RR (Supplementary Fig. 5). These results may exclude the possibility that the results of azilsartan were mediated by changes in pressure or volume overload on the heart in a nonspecific 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 (Supplementary Fig. 6) but did not affect the expression of cardiac (P)RR (Supplementary Fig. 7). This suggests that the effect of azilsartan in decreasing the expression of cardiac (P)RR is not a class effect of ARBs but a peculiar effect of azilsartan.

Clinical implications

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

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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