Cardiovascular Drugs and Therapy
https://doi.org/10.1007/s10557-019-06900-1

ORIGINAL ARTICLE

Angiotensin II Type 1 Receptor Antagonist Azilsartan Restores Vascular Reactivity Through a Perivascular Adipose Tissue-Independent Mechanism in Rats with Metabolic Syndrome

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Abstract

Purpose Perivascular adipose tissues (PVAT) are involved in the regulation of vascular tone. In mesenteric arteries, the compensatory vasodilatory effects of PVAT appear when vascular relaxation is impaired and disappear at around 23 weeks of age in SHRSP.Z-*Lepr*^{fa}/IzmDmcr (SHRSP.ZF) rats with metabolic syndrome (MetS). The renin-angiotensin system is involved in the development of endothelium and vascular dysfunction. Therefore, we investigated whether azilsartan, a potent angiotensin II type 1 (AT1) receptor antagonist, can protect against the deterioration of the PVAT compensatory vasodilator function that occurs with aging in MetS.

Methods Two age groups of SHRSP.ZF rats (13 and 20 weeks of age) were administered azilsartan or vehicle through oral gavage once daily for 10 weeks. The vasodilation response of the isolated superior-mesenteric arteries upon addition of endothelium-dependent and -independent agonists was determined in the presence or absence of PVAT using organ bath methods.

Results In vivo treatment with azilsartan improved the acetylcholine-induced vasodilation in mesenteric arteries with and without PVAT at both time-points. The mRNA levels of AT1 receptor and AT1 receptor-associated protein were unchanged in PVAT upon azilsartan treatment. Furthermore, in vitro treatment with azilsartan (0.1 and 0.3 μM for 30 min) did not affect the compensatory effect of PVAT on vasodilation in response to acetylcholine in SHRSP.ZF rat mesenteric arteries.

Conclusions Our results provide evidence supporting the use of azilsartan for the long-term protection against vascular dysfunctions in MetS. Azilsartan did not improve the dysfunction of PVAT-mediated modulation of vascular tone during MetS. The protective effect of azilsartan is mediated by restoring the endothelium- and vascular smooth muscle-mediated mechanisms.

Keywords Adipose tissue · AT1 receptor · Angiotensin II · Azilsartan · Metabolic syndrome · Vasodilation

Introduction

Metabolic syndrome (MetS) facilitates the development of cardiovascular disease due to atherosclerosis. The symptoms of MetS, a chronic condition, worsen with age. Thus, MetS requires a long-term clinical treatment strategy. Angiotensin II type 1 (AT1) receptor antagonists are widely used as therapeutic agents against MetS, as they are effective against a wide range of MetS symptoms [1, 2]. Azilsartan is a potent long-acting antagonist of the AT1 receptor that has a favorable safety profile and is used in the treatment of hypertension with once-daily dosing [3–5]. Azilsartan has a high affinity to the AT1 receptor. The vascular concentration of azilsartan, rather than the plasma concentration, elicits the blood pressure-lowering effect through inhibition of vascular AT1 receptors [6]. Azilsartan is reported to exhibit beneficial preventative/therapeutic effects against cardiovascular dysfunction by mitigating the vascular endothelium dysfunction and by lowering the blood pressure in patients with MetS and diabetes. Azilsartan is reported to lower the blood pressure and improve the vascular endothelium function in SHR/NDmcr-cp rats [7] and spontaneously hypertensive obese rats [8] with MetS. Additionally, azilsartan is reported to protect

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10557-019-06900-1) contains supplementary material, which is available to authorized users.

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Published online: 16 August 2019

against renal dysfunction in Zucker diabetic fatty rats with type 2 diabetes by lowering the blood pressure and exhibiting antioxidant activity [9]. Azilsartan exhibits depressor and renoprotective effects in patients with hypertension [10]. Azilsartan also exhibits preventive and therapeutic vasculoprotective activity on diabetes-induced cerebrovascular remodeling in Goto-Kakizaki rats with type 2 diabetes independent of the blood pressure-lowering effects [11]. These studies suggest that azilsartan may have other therapeutic effects against MetS in addition to its blood pressure-lowering effect.

Perivascular adipose tissue (PVAT), which is located outside the blood vessels, can regulate the vascular homeostasis by releasing vasoactive molecules [12, 13]. PVAT dysfunction is associated with the development of vascular complications. Hence, improving PVAT functions can be a preventive therapeutic strategy against vascular disease. Our previous study demonstrated that chronic oxidative/nitrative stress is associated with the development of vascular dysfunction in response to nitric oxide (NO) in the coronary and mesenteric arteries of SHRSP.Z-*Lepr*^{fa}/IzmDmcr (SHRSP.ZF) rats [14], which is an animal model of MetS [15, 16]. Recently, we reported that the PVAT exhibits a compensatory effect on the impaired NO-dependent vasodilation in the mesenteric arteries of SHRSP.ZF rats (17 and 20 weeks of age), and that this compensatory effect is diminished at later stages of MetS (23 and 30 weeks of age) [17, 18]. By contrast, the mesenteric arterial PVAT of Wistar-Kyoto rats, a non-diseased (healthy) control, did not exhibit any effect on the vasodilation at 10, 20, and 30 weeks of age [17]. We propose that the enhanced activity of the renin-angiotensin system in PVAT contributes to the vascular dysfunction upon long-term continuous exposure to high blood pressure and metabolic abnormalities [18]. The rat aortic and mesenteric adipose tissues comprise essential components of the renin-angiotensin system, including AT1 receptors and angiotensin II [12, 19]. These findings suggest that the inhibition of renin-angiotensin system can be a pharmaceutical therapeutic strategy against PVAT function impairment in SHRSP.ZF rats associated with aging. However, there are limited studies that have evaluated the effect of AT1 antagonists on the regulation of vasodilation by the PVAT compensatory system under pathophysiological conditions of MetS. Therefore, the aim of this study was to examine the protective effect of azilsartan against the age-related impairment of PVAT-mediated vasodilation in SHRSP.ZF rats at two different time-points (23 and 30 weeks of age), which represent the different phenotypes in the progression of MetS.

Materials and Methods

Drugs

Azilsartan used for in vivo treatment was provided by Takeda Pharmaceutical Co. Ltd. (Tokyo, Japan). Additional reagents and chemicals were also purchased from suppliers: azilsartan (FUJIFILM Wako Pure Chemical Co., Osaka, Japan), L-phenylephrine hydrochloride (Sigma-Aldrich Co., LLC., St. Louis, USA), acetylcholine chloride (Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan), and sodium nitroprusside (Nacalai Tesque Inc., Kyoto, Japan). Other chemicals of analytical reagent grade were purchased from Nacalai Tesque Inc. (Kyoto, Japan).

Experimental Animals

All protocols involving the care and use of animals were approved by the animal ethics committee and performed in accordance with the Guidelines for the Care and Use of Laboratory Animals at Mukogawa Women's University (protocol numbers: P-12-2016-01-A, P-12-2017-01-A, and P-12-2018-01-A).

Thirty-six male SHRSP.ZF rats, a strain established by the Disease Model Cooperative Research Association, aged 12 and 19 weeks old ($n = 18$ at each age) were purchased from Japan SLC, Inc. (Hamamatsu, Japan). After the rats were allowed to habituate for 1 week, systolic blood pressure and body weight of each rat were examined at baseline (T_0). The rats were randomly assigned to three groups ($n = 6$ per group: control, low-dose azilsartan, and high-dose azilsartan). The treatment was started at 13 or 20 weeks of age. The SHRSP.ZF rats were treated with vehicle (control, 0.5% methylcellulose solution 0.1 mL/100 g body weight) or azilsartan (1 mg azilsartan/kg body weight or 3 mg azilsartan/kg body weight) by oral gavage once daily for 10 weeks consecutively. The age of the animals was selected based on the findings of our earlier studies, which demonstrated that the breakdown of PVAT compensatory system occurs at 23 and 30 weeks of age in rats [17, 18]. The azilsartan treatment protocol was based on previous studies on MetS model rats [7, 8, 20], hypertension model rats [6], and diabetic model rats [11]. The systolic blood pressure was assessed at $T_0 + 9$ weeks. At $T_0 + 10$ weeks, the blood samples were drawn for assessing the serum metabolites and an oxidative stress biomarker. The vascular function was assessed as described below. For in vitro treatment with azilsartan, the SHRSP.ZF rats aged 5 weeks old ($n = 9$) were purchased from Japan SLC, and the vascular function of each rat was assessed at 21–23 weeks of age. The rats were fed with a standard chow (CE-2; Clea Japan Inc., Tokyo, Japan) and water ad libitum during the experimental period.

Determination of Metabolic Parameters

Systolic arterial blood pressure was measured by a tail-cuff method (MK-2000; Muromachi, Tokyo, Japan) between 9 am and 12 pm before the daily dose of drug was administered as described previously [21]. The average systolic blood pressure value was obtained from at least five measurements per animal at each time-point. The body weight was measured after anesthetizing the animal with ketamine (90 mg/kg body

weight, i.p.) and xylazine (10 mg/kg body weight, i.p.). Next, the blood samples were collected directly from the abdominal aorta of rats. The blood sample was centrifuged for 10 min at 3000×*g* and 4 °C. The serum levels of thiobarbituric acid reactive substances (TBARS), which is an indicator of oxidative stress, were determined using the commercial kit purchased from Cayman Chemical Co. (Ann Arbor, MI, USA).

Determination of Vasodilation

Bioassays of vascular function were conducted as we described previously [17]. Briefly, superior-mesenteric arteries were excised from each rat under anesthesia after blood collection (described above). The arteries were immediately placed in Krebs-Henseleit (Krebs) buffer (pH 7.4; NaCl 118.4 mM, KCl 4.7 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 2.5 mM, $NaHCO_3$ 25 mM, KH_2PO_4 1.2 mM, and glucose 11.1 mM) and saturated by bubbling with 95% O_2 and 5% CO_2 gas mixture. The arteries with or without intact PVAT were cut into 3-mm rings and mounted isometrically at an optimal resting tension (0.3 g) in 10 mL organ baths filled with Krebs buffer. After an equilibrium period at resting tension, we conducted a viability protocol as follows. Three micromolars of phenylephrine was added to each artery, and then, after reaching a stable contraction, 10 μM acetylcholine was added to each artery. Tissues were excluded from further experimentation if they failed to contract with phenylephrine or relax with acetylcholine. Following the viability protocol, cumulative phenylephrine concentration dose-response curves were determined for each artery. After a washout period, ring preparations of mesenteric arteries were contracted to ~80% of their maximal contractions via addition of 2 μM phenylephrine, and then, relaxation dose-response curves were constructed by addition of cumulative increasing concentrations of agonists. The dose-response curves were generated for acetylcholine (0.1 nM–1 μM) and sodium nitroprusside (0.1 nM–1 μM). For in vitro treatment with azilsartan, the same vascular bioassays were performed in the presence of azilsartan (0.1 and 0.3 μM, 30 min) or vehicle (dimethyl sulfoxide, 10 μL) using isolated arteries from SHRSP.ZF rats, which were not administered with azilsartan. The in vitro treatment condition for azilsartan was based on previously published EC_{50} (9.2 nM) and pD'_2 (9.9) values [5].

Isometric tension changes were measured with a force-displacement transducer (Model t-7; NEC San-Ei, Tokyo, Japan) coupled to a dual channel chart recorder (Model 8K21; NEC San-Ei). The stock solutions of test compounds were prepared in distilled water. A relaxation response (% relaxation) at each dose of vasodilator was derived from the percent reversal of the precontraction tension elicited by 2 μM phenylephrine, i.e., 100% represents complete return to resting tension. For each vasodilator drug, the dose-response curves were analyzed by nonlinear regression to fit a sigmoidal (constant Hill slope = 1) dose-response curve and calculate corresponding negative log EC_{50} (a measure of sensitivity) and E_{max} (a measure of maximal response) values for each artery using GraphPad Prism® (ver. 5.0, San Diego, CA, USA).

Determination of mRNA Expression

Quantitative real-time polymerase chain reaction (qRT-PCR) assay was performed to examine the mRNA expression levels of AT1 receptor, AT1 receptor-associated protein (ATRAP), and angiotensinogen in PVAT of SHRSP.ZF rats orally treated with azilsartan for 10 weeks at 23 and 30 weeks of age, as described previously [18]. The qRT-PCR analysis was performed using TaqMan RNA-to-CT one-step kit and LightCycler 1.5 (Roche Diagnostics Japan K.K., Tokyo, Japan). The mRNA expression levels in the sample were normalized using the expression levels of housekeeping gene (ribosomal protein 18S, β- glucuronidase, and β-actin). The efficiency of primer sets was included in all calculations. The target gene expression level was normalized to the reference gene expression level to obtain the relative threshold cycle (ΔC_T) and then to the C_T of the control group to obtain the relative expression level ($2^{-\Delta\Delta CT}$) of target gene. The data were combined from three independent experiments. The ratio of AT1 receptor to ATRAP expression was calculated as an index of AT1 receptor activity.

We used the following commercially available gene-specific probes from Roche Applied Science (numbers represent the Universal Probe Library product identifiers): AT1 receptor, 04688503001; ATRAP, 04684982001; angiotensinogen, 04685059001; ribosomal protein 18S, 04688937001; β-glucuronidase, 04688015001; β-actin, 04686900001). Gene-specific primers designed in Assay Design Center (Roche Applied Science) were purchased from Life Technologies Japan Ltd. (Tokyo, Japan).

Statistical Analysis

Data are expressed as mean ± standard error of mean (SEM). The means between the groups were analyzed using Student's *t* test or one-way analysis of variance (ANOVA) followed by the Bonferroni post-hoc test. The differences were considered statistically significant when the *P* value was less than 0.05.

Results

Effect of In vivo Treatment with Azilsartan on Metabolic Parameters of SHRSP.ZF Rats

As shown in Table 1, the once-daily treatment with azilsartan for 10 weeks initiated at different time-points (13 weeks or

Table 1 Effects of in vivo treatment with azilsartan on body weight, systolic blood pressure, and serum thiobarbituric acid reactive substances (TBARS) levels in SHRSP.Z-*Lepr*^{fa}/IzmDmcr (SHRSP.ZF) rats at 23 and 30 weeks of age (weeks)

	Group (*n*)	Body weight (g)	Systolic blood pressure (mmHg)	TBARS (μM)
Early stage (23 weeks)	Control (6)	489 ± 11	256 ± 6	17.4 ± 2.2
	Low-dose azilsartan (6)	485 ± 7	203 ± 6*	12.7 ± 2.1
	High-dose azilsartan (6)	464 ± 7	177 ± 4$^{*\#}$	12.4 ± 1.5
Later stage (30 weeks)	Control (6)	451 ± 19	274 ± 7	15.5 ± 1.4
	Low-dose azilsartan (6)	481 ± 6	209 ± 3*	16.5 ± 1.0
	High-dose azilsartan (6)	474 ± 6	178 ± 8$^{*\#}$	9.69 ± 0.6$^{*\#}$

Results are expressed as the mean ± SEM. SHRSP.ZF rats were administered vehicle (control, 0.5% methylcellulose solution 1 mL/kg body weight), 1 mg/kg azilsartan (low-dose azilsartan), or 3 mg/kg azilsartan (high-dose azilsartan) by oral gavage daily for 10 weeks from 13 (early stage) and 20 weeks (later stage), respectively. $^{*}P < 0.05$, as compared with each control, $\#P < 0.05$, as compared with each low-dose group

20 weeks) did not significantly affect the body weight of SHRSP.ZF rats. Azilsartan treatment resulted in a dose-dependent reduction of systolic arterial blood pressure in both experimental age groups. The TBARS levels decreased significantly in the high-dose azilsartan treatment group at later stages of MetS.

Effect of In vivo Treatment with Azilsartan on Vascular Functions of Superior-Mesenteric Arteries in SHRSP.ZF Rats

The effect of azilsartan on vascular function was assessed in SHRSP.ZF rats by measuring the PVAT-mediated enhanced endothelium-dependent vasodilation (Figs. 1 and 2) and endothelium-independent vasodilation (supplementary Figs. 1 and 2) at different ages. Figure 1 and Table 2 show improvement of endothelium-dependent vasodilation in 23-week-old SHRSP.ZF rats after azilsartan treatment (once-daily dose for 10 weeks). In vivo azilsartan treatment enhanced the relaxation of superior-mesenteric arteries without PVAT (Fig. 1a) and that of arteries with PVAT (Fig. 1b). Furthermore, there were no significant differences between the relaxation response of arteries with intact PVAT and that of arteries without intact PVAT in all experimental groups (Fig. 1c–e). Moreover, similar enhancements in the relaxation response were observed between the mesenteric arteries with PVAT and those without PVAT in SHRSP.ZF rats administered with azilsartan for 10 weeks, whose treatment was initiated at 20 weeks of age (Fig. 2 and Table 2).

As observed in acetylcholine-induced relaxation, azilsartan treatment also enhanced the sodium nitroprusside-induced relaxation (endothelium-independent nitric oxide-mediated responses of vascular smooth muscle cells) in mesenteric arteries with or without intact PVAT in all treatment groups at two different ages of SHRSP.ZF rats (supplementary Figs. 1 and 2, and supplementary Table 1).

Effect of In vivo Treatment with Azilsartan on mRNA Expression Levels in Mesenteric PVAT of SHRSP.ZF Rats

Previously, we had reported that the mRNA levels of ATRAP and angiotensinogen decreased, while those of the AT1 receptor remained unchanged in the mesenteric PVAT of SHRSP.ZF rats when the PVAT-mediated enhanced vasodilation was impaired during aging [18]. In this study, the mRNA expression levels of the AT1 receptor, ATRAP, and angiotensinogen were not significantly different between the drug treatment groups (Fig. 3a–d and g, h). Additionally, the ratios of AT1 receptor to ATRAP expression were not different between the groups (Fig. 3e, f).

Effect of In vitro Treatment with Azilsartan on Vascular Functions of Isolated Superior-Mesenteric Arteries in SHRSP.ZF Rats

In the presence of PVAT, acetylcholine-induced relaxation was enhanced in 21 to 23-week-old SHRSP.ZF rats, but theses relaxations were unaffected by in vitro pretreatment with 0.1 or 0.3 μM azilsartan (Fig. 4 and Table 3).

Discussion

Vasodilation is impaired during high blood pressure, metabolic abnormalities, and enhanced oxidative stress induced by angiotensin II in the superior-mesenteric arteries of SHRSP.ZF rats with MetS [14]. The arteries are enclosed by PVAT, which helps in the regulation of vasodilation to compensate for the impaired mechanisms under pathophysiological conditions [17]. However, the compensatory function of PVAT is lost at later stages of MetS (23 and 30 weeks of age) [17, 18]. This study demonstrated that administering azilsartan preserves not only the endothelium-dependent acetylcholine-

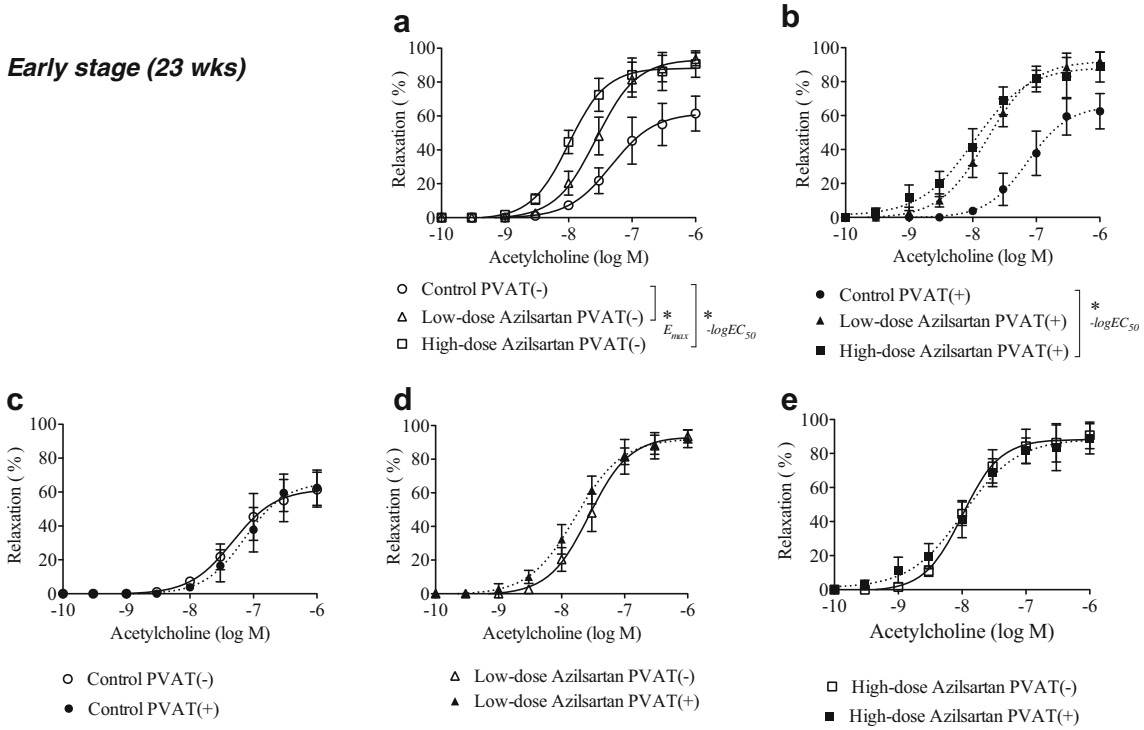

Fig. 1 Vasodilation in response to acetylcholine in superior-mesenteric arteries without (**a**) or with (**b**) perivascular adipose tissue (PVAT) from SHRSP.Z-*Lepr*^{fa}/IzmDmcr (SHRSP.ZF) rats at 23 weeks of age. SHRSP.ZF rats were administered with vehicle (0.5% methylcellulose solution 1 mL/kg body weight, control, **c**), 1 mg/kg azilsartan (low-dose azilsartan, **d**), or 3 mg/kg azilsartan (high-dose azilsartan, **e**) by oral gavage daily for 10 weeks from 13 weeks of age. *$P < 0.05$

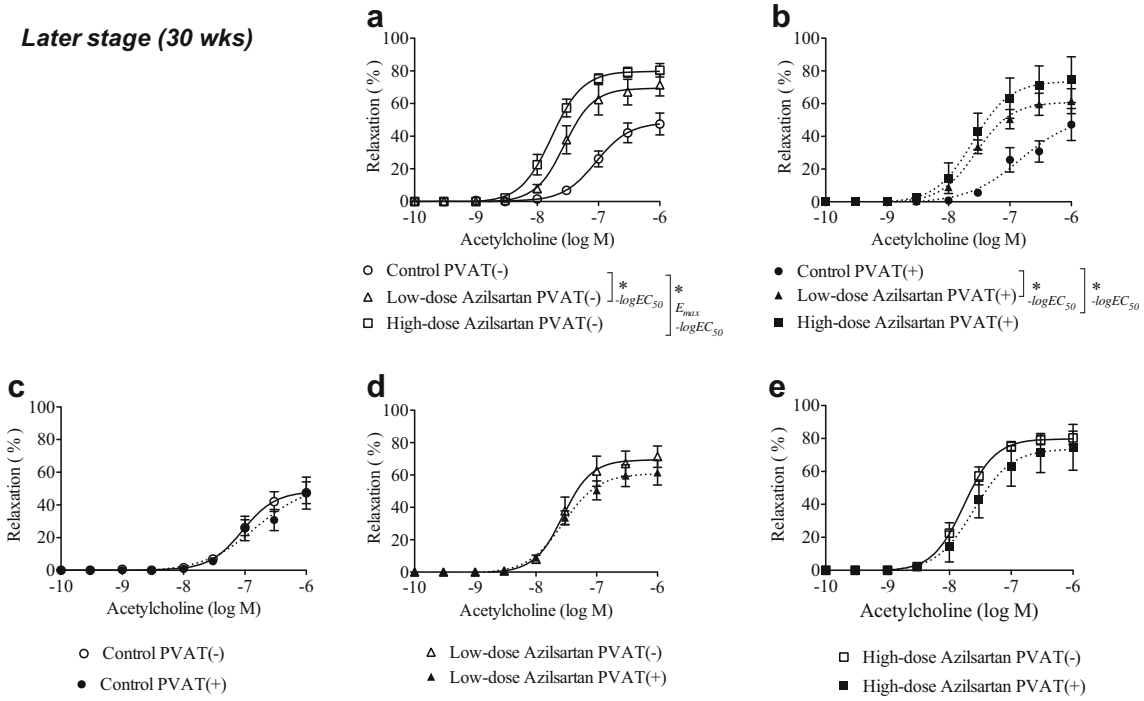

Fig. 2 Vasodilation in response to acetylcholine in superior-mesenteric arteries without (**a**) or with (**b**) perivascular adipose tissue (PVAT) from SHRSP.Z-*Lepr*^{fa}/IzmDmcr (SHRSP.ZF) rats at 30 weeks of age. SHRSP.ZF rats were administered with vehicle (0.5% methylcellulose solution 1 mL/kg body weight, control, **c**), 1 mg/kg azilsartan (low-dose azilsartan, **d**), or 3 mg/kg azilsartan (high-dose azilsartan, **E**) by oral gavage daily for 10 weeks from 20 weeks of age. *$P < 0.05$

Table 2 Effects of in vivo treatment with azilsartan on relaxations in response to acetylcholine in isolated superior-mesenteric arteries with (+) or without (−) perivascular adipose tissue (PVAT) from SHRSP.Z-*Lepr*^{fa}/IzmDmcr (SHRSP.ZF) rats at 23 and 30 weeks of age (weeks)

	Group (*n*)		Acetylcholine	
			-Log EC_{50}	E_{max}
Early stage (23 weeks)	Control (6)	PVAT(−)	7.08 ± 0.23	61.4 ± 10.3
		PVAT(+)	6.98 ± 0.22	62.6 ± 10.4
	Low-dose azilsartan (6)	PVAT(−)	7.47 ± 0.20	93.5 ± 3.8*
		PVAT(+)	7.79 ± 0.12	92.2 ± 5.3
	High-dose azilsartan (6)	PVAT(−)	7.91 ± 0.11*	90.7 ± 7.8
		PVAT(+)	8.00 ± 0.23*	88.6 ± 8.8
Later stage (30 weeks)	Control (6)	PVAT(−)	7.03 ± 0.10	45.5 ± 4.2
		PVAT(+)	6.83 ± 0.28	52.5 ± 13.0
	Low-dose azilsartan (6)	PVAT(−)	7.56 ± 0.08*	69.4 ± 3.9
		PVAT(+)	7.55 ± 0.08*	60.9 ± 3.3
	High-dose azilsartan (6)	PVAT(−)	7.71 ± 0.04*	80.7 ± 2.1*
		PVAT(+)	7.56 ± 0.34*	73.0 ± 13.3

Results are expressed as the mean ± SEM. SHRSP.ZF rats were administered vehicle (control, 0.5% methylcellulose solution 1 mL/kg body weight), 1 mg/kg azilsartan (low-dose azilsartan), or 3 mg/kg azilsartan (high-dose azilsartan) by oral gavage daily for 10 weeks from 13 (early stage) and 20 weeks (later stage), respectively. *$P < 0.05$, as compared with each control

mediated relaxation but also the endothelium-independent sodium nitroprusside-mediated vasodilation in SHRSP.ZF rat artery at both early and later stages of MetS. We propose that the underlying mechanism of azilsartan-mediated improved response to NO in the vascular smooth muscle cells involves lowering blood pressure and antioxidant effects. Interestingly, azilsartan did not preserve the PVAT-mediated modulation of vasodilation in aged SHRSP.ZF rats. This suggested that the impaired PVAT compensatory function is not mediated by the AT1 receptors and that the vascular function can be restored independent of PVAT compensatory function.

Azilsartan treatment improved NO-mediated vasodilation in SHRSP.ZF rats at both time-points in SHRSP.ZF rats. Other AT1 receptor antagonists, such as telmisartan and olmesartan, can prevent impaired relaxation in mesenteric arteries by protecting the arteries against high blood pressure and oxidative stress in SHRSP.ZF rats [14, 22]. Furthermore, our data indicated that azilsartan reduces the oxidative stress levels. This suggested that azilsartan affects the artery's response to NO and improves the endothelium-dependent vasodilation at later stages of MetS. TBARS levels are an indicator of systemic lipid peroxidation. Hence, local oxidative stress mechanisms must be evaluated using a tissue-selective marker of oxidative stress. Our results concur with those of our previous studies that indicated a correlation between elevated levels of oxidative stress in SHRSP.ZF rats and decreased expression of soluble guanylyl cyclase protein [14], which is a key enzyme for NO-mediated vasodilation. Consistent with our findings, azilsartan was reported to prevent endothelial dysfunction in diabetic mice by exerting a dual effect on uncoupled endothelial NO synthase and on NAD(P)H oxidase in the aorta of KKAy diabetic mice [23]. Similarly, azilsartan improves acetylcholine-induced relaxation in mesenteric arteries of spontaneously hypertensive obese rats with MetS [8]. The underlying mechanism involved in the azilsartan-mediated vasodilation involves enhanced phosphorylation of the enzyme in the aorta of KKAy diabetic mice [23]. Hence, azilsartan may mitigate the endothelial dysfunctions in MetS. Validating the mechanisms of a class effect of AT1 blockers on protecting the endothelium warrants continued studies using azilsartan in MetS models and different vascular tissues.

Although azilsartan lowered the blood pressure and reduced the levels of an oxidative stress marker at later age, the compensatory function of PVAT was not restored upon treatment with azilsartan. This concurred with an earlier study, which reported that treatment with losartan (10 mg/kg body weight for 8 weeks) does not improve the impaired PVAT-mediated modification of acetylcholine-induced relaxation in the aorta of fructose-fed rats [24]. These findings suggest that inhibition of AT1 receptors is sufficient to restore the vascular tone, which does not require the mitigation of impaired PVAT compensatory functions. Previously, we reported that PVAT compensatory functions were not observed in younger SHRSP.ZF rats (10 weeks of age), where the vascular relaxation functions were maintained at physiological levels [17]. We propose that the compensatory functions of PVAT are exhibited only during impaired artery's response to NO for restoring or maintaining the vascular function.

Several studies have reported that administering AT1 antagonist affects the AT1 receptor expression in adipocytes. Wang et al. reported that treatment with losartan (30 mg/kg/

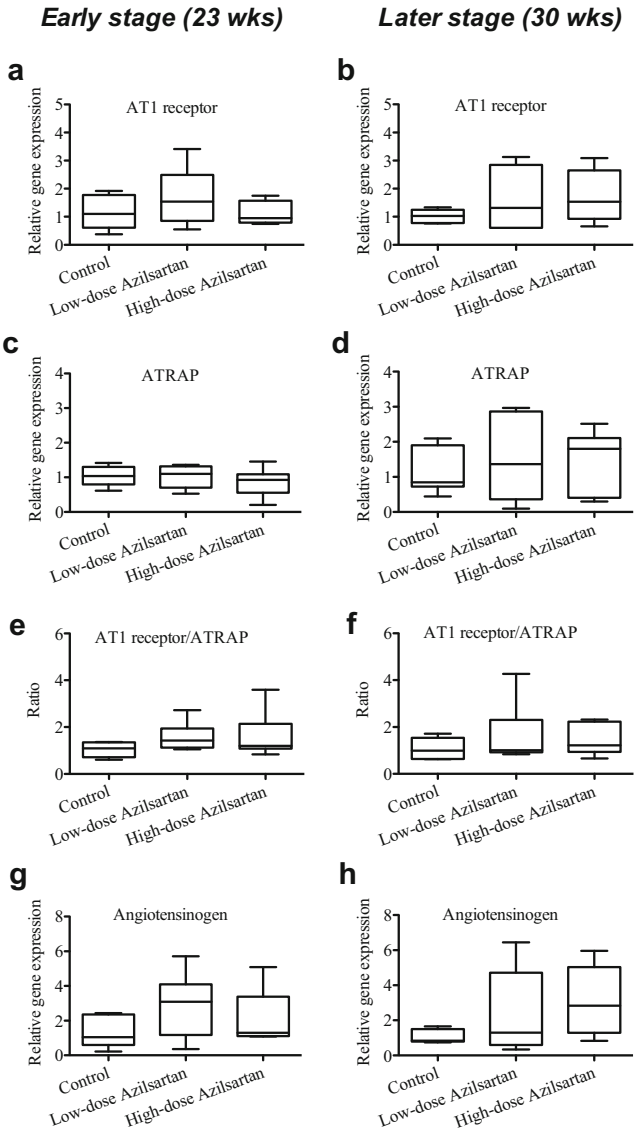

Fig. 3 The mRNA transcript levels of angiotensin II type 1 (AT1) receptor (**a** and **b**), AT1 receptor-associated protein (ATRAP, **c** and **d**), and angiotensinogen (**g** and **h**) in superior-mesenteric arterial perivascular adipose tissue (PVAT) of SHRSP.Z-*Lepr*^{fa}/IzmDmcr (SHRSP.ZF) rats at 23 and 30 weeks of age and the ratio of AT1 receptor to ATRAP expression (**e** and **f**). SHRSP.ZF rats were administered with vehicle (0.5% methylcellulose solution 1 mL/kg body weight, control), 1 mg/kg azilsartan (low-dose azilsartan), or 3 mg/kg azilsartan (high-dose azilsartan) by oral gavage daily for 10 weeks from 13 and 20 weeks of age

day for 16 weeks) resulted in mitigation of enhanced AT1 mRNA levels in the adipocytes of high-fat diet-fed spontaneously hypertensive rats [25]. Maeda et al. reported that treatment with irbesartan (50 mg/kg/day for 4 weeks) decreased the AT1 mRNA levels, while treatment with olmesartan (3 mg/kg/day for 4 weeks) increased the AT1 mRNA levels in adipocytes of KKAy mice [26]. Recently, we reported that mRNA levels of the AT1 receptor in the mesenteric PVAT of SHRSP.ZF rats were higher at 20 weeks of age (when the compensatory PVAT effects on vasodilation begins) when

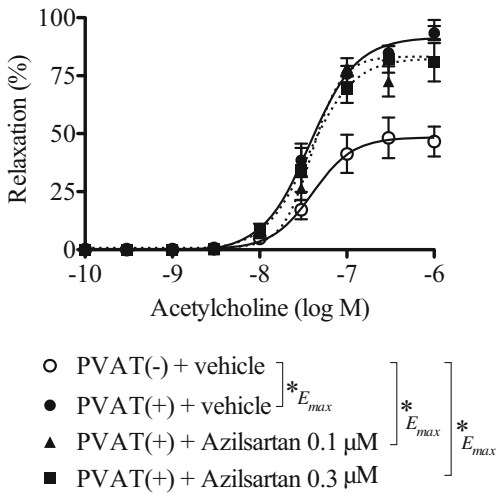

Fig. 4 Vasodilation in response to acetylcholine in superior-mesenteric arteries with (+) or without (−) perivascular adipose tissue (PVAT) from SHRSP.Z-*Lepr*^{fa}/IzmDmcr (SHRSP.ZF) rats at 21–23 weeks of age. Before inducing contraction-relaxation term, the isolated arterial preparations were treated with 0.1 or 0.3 μM azilsartan or vehicle (dimethyl sulfoxide, 10 μL) for 30 min, respectively. *$P < 0.05$

compared to those in the mesenteric PVAT of age-matched Wistar-Kyoto rats (normal control) [18]. By contrasts, the AT1 receptor levels remained unaltered, while the ATRAP levels were lower in SHRSP.ZF at 23 weeks of age when compared to those at 20 weeks of age. This suggested that the AT1 receptor activity is enhanced in mesenteric PVAT with age [18]. ATRAP, which interacts with the AT1 receptor, suppresses the angiotensin II-mediated AT1 receptor signaling [27]. The expression of ATRAP in adipose tissues decreases in patients and mice with MetS [28]. The higher ratio of ATRAP/AT1 receptor mRNA expression was proposed to be the underlying mechanism that mediates the effects of irbesartan and losartan on adipose tissue in genetically obese diabetic KKAy mice [29], and high-fat diet-fed spontaneously hypertensive rats [25], respectively. However, this study revealed that the chronic inhibition of AT1 receptor activity by

Table 3 Effects of in vitro treatment with azilsartan on relaxations in response to acetylcholine in isolated superior-mesenteric arteries with (+) or without (−) perivascular adipose tissue (PVAT) from SHRSP.Z-*Lepr*^{fa}/IzmDmcr (SHRSP.ZF) rats at 21–23 weeks of age (weeks)

Group (*n*)	Acetylcholine	
	-Log EC_{50}	E_{max}
PVAT(−) + vehicle (9)	7.20 ± 0.21	55.5 ± 8.0
PVAT(+) + vehicle (9)	7.43 ± 0.07	91.5 ± 3.3*
PVAT(+) + azilsartan 0.1 μM (9)	7.37 ± 0.05	85.8 ± 5.6*
PVAT(+) + azilsartan 0.3 μM (9)	7.42 ± 0.06	82.3 ± 7.0*

Results are expressed as the mean ± SEM. Before contraction-relaxation term, isolated mesenteric arteries from SHRSP.ZF rats were treated with vehicle (dimethyl sulfoxide, 10 μL), 0.1, or 0.3 μM azilsartan for 30 min, respectively. *$P < 0.05$, as compared PVAT(−)

administering a potent AT1 antagonist, such as azilsartan, did not alter the mRNA levels of AT1 receptor and ATRAP as well as the AT1 receptor/ATRAP ratio in PVAT at both 23 and 30 weeks of age. The varied responses to AT1 receptor antagonists on the components of the renin-angiotensin system in adipose tissue may be due to the differences in the specific drug, animal model, and disease pathologies used in the study.

Initially, we speculated that the activation of AT1 receptors by angiotensin II initiates the deterioration of the adaptive responses of PVAT, which involves modification of vascular tone by PVAT during impaired vasorelaxation in MetS. However, in vitro treatment with azilsartan did not affect the enhanced acetylcholine-induced relaxation in the presence of PVAT. This suggests that AT1 receptor activation in PVAT is not directly involved in PVAT-mediated enhanced vasodilation in SHRSP.ZF rats with MetS. Other studies have reported that adipocyte-derived angiotensin II potentiates the contraction of rat mesenteric artery in normal rats [30]. However, our data does not concur with this mechanism. The anti-contractile effects of PVAT are mediated by AT2 receptors in the thoracic aorta of normal mice [31]. Further studies are needed to assess the role of AT2 receptor in mesenteric PVAT of SHRSP.ZF rats. The angiotensin II receptors are reported to exhibit a functional role in browning of white adipose tissue. Tsukuda et al. have demonstrated that the deletion of AT1a receptor, and not AT2 receptor, results in the suppression of adipocyte differentiation in white adipose tissue of the inguinal region [32]. A recent study reported that the decrease in PVAT browning with aging results in attenuated vasorelaxing effect on the thoracic aorta in spontaneously hypertensive rats [33]. These findings indicate that azilsartan-mediated inhibition of AT1 in PVAT induces decreased mesenteric PVAT browning, which results in loss of a compensatory effect of PVAT in SHRSP.ZF rats with MetS.

In conclusion, azilsartan preserved vasodilation through a mechanism that included improved vascular smooth muscle cells response to NO in SHRSP.ZF rats at later stages of MetS. However, the dysfunction of PVAT was not mitigated by azilsartan in adipose tissue of SHRSP.ZF rats. Hence, azilsartan may repair vascular dysfunctions and not PVAT dysfunction at later stages of MetS.

Acknowledgments Azilsartan used in in vivo treatment was a generous gift from Takeda Pharmaceutical Co. Ltd. (Tokyo, Japan). The authors wish to thank Ms. Saki Iwata, Ms. Rui Yamada, Ms. Maho Mizuno, Ms. Shiori Koyanagi, and Ms. Yayoi Shiokawa in Mukogawa Women's University for their technical support.

Author Contributions Conceptualization of study, Satomi Kagota; Experimental design, Satomi Kagota; Performed the experiments and data analysis, Satomi Kagota, Kana Maruyama-Fumoto, and Miho Shimari; Data interpretation, Satomi Kagota and John J. McGuire; Funding acquisition and supervision, Satomi Kagota and Kazumasa Shinozuka; Original manuscript draft preparation, Satomi Kagota and John J. McGuire; Reviewing and editing the manuscript, Satomi Kagota and John J. McGuire. All authors have read and approved the final manuscript.

Funding Information This work was partly supported by JSPS KAKENHI (grant number JP16K08563) to SK.

Compliance with Ethical Standards

This study was approved by the Animal Experimentation Committee at Mukogawa Women's University (protocol numbers: P-12-2016-01-A, P-12-2017-01-A, and P-12-2018-01-A), and all animal experiments were performed in accordance with the guidelines for the Care and Use of Laboratory Animals at Mukogawa Women's University.

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

Research Involving Animals All protocols involving the care and use of animals were approved by the animal ethics committee and performed in accordance with the Guidelines for the Care and Use of Laboratory Animals at Mukogawa Women's University (protocol numbers: P-12-2016-01-A, P-12-2017-01-A, and P-12-2018-01-A).

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