ORIGINAL ARTICLE

Significance of angiotensin II receptor blockers with high affinity to angiotensin II type 1 receptors for vascular protection in rats

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Angiotensin II receptor blockers (ARBs) vary in their binding affinities to angiotensin II type 1 (AT₁) receptors in *in vitro* experiments. We compared a high-affinity ARB, olmesartan, and a low-affinity ARB, valsartan, in terms of their vascular protective effects in stroke-prone spontaneously hypertensive rats (SHR-SP). Blood pressure was equally reduced by placebo, olmesartan (1 mg kg⁻¹) and valsartan (3 mg kg⁻¹) daily for 2 weeks. In another experiment, 12-week-old SHR-SP were fed 8% salt, and olmesartan (1 mg kg⁻¹), valsartan (3 mg kg⁻¹) or placebo were administered daily until a survival rate of 60% was reached. In the experiment using SHR-SP, the reduction of acetylcholine-induced vascular relaxation and the increase of p22^{phox} expression in the placebo-treated group were significantly attenuated by olmesartan and valsartan, but this attenuation was significantly greater for olmesartan. In immunohistological analysis, all areas positive for angiotensin II, p22^{phox} and 4-hydroxy-2-nonenal were significantly reduced by olmesartan and valsartan, but again this reduction was significantly greater for olmesartan. In salt-loaded SHR-SP, the number of days to reach a 60% survival rate was 25 and 42 in placebo and valsartan-treated rats, respectively, and this represented a significant difference. The survival rate in olmesartan-treated rats was 95% at day 42, when valsartan-treated rats reached 60% survival, and this difference was also significant. In the surviving rats, olmesartan, but not valsartan, augmented acetylcholine-induced vascular relaxation and attenuated vascular p22^{phox} expression. Thus, heterogeneity in binding affinity to AT₁ receptors among ARBs may result in different degrees of vascular protection and lifespan extension.

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INTRODUCTION

Angiotensin II has an important role in increasing blood pressure by stimulating angiotensin II type 1 (AT₁) receptors. Stimulation of these receptors by angiotensin II also induces growth factors and cytokines that have important roles in cardiovascular disease.¹ Therefore, in blocking the binding of angiotensin II to AT₁ receptors, angiotensin II receptor blockers (ARBs) not only reduce blood pressure but also prevent cardiovascular dysfunction. Angiotensin-converting enzyme (ACE) inhibitors have also been widely applied in the clinical setting and work by inhibiting angiotensin II formation. In many clinical studies, both ARBs and ACE inhibitors have shown greater prevention of cardiovascular dysfunction than other antihypertensive drugs. However, within each of these classes of drugs, cardioprotective effects may vary. Pilote *et al.*² conducted several randomized controlled trials to evaluate the effects on survival of seven ACE inhibitors in patients with acute myocardial infarction; they found that the survival extension varied considerably among these agents. We previously compared four ACE inhibitors, enalapril, temocapril, perindopril and trandola-

pril, at doses that showed the same hypotensive effects, in terms of cardiovascular ACE inhibition in hypertensive rats.³ The degree of ACE inhibition varied considerably among these ACE inhibitors, and we showed a significant correlation between cardiovascular ACE inhibition and the degree of cardioprotection conferred.³ Thus, ACE inhibitors that strongly inhibit tissue ACE activity are more effective than those that do so weakly, even using doses that give the same hypotensive effects. Similarly, there may also be differences in cardiovascular protection among ARBs, even using doses with the same hypotensive effect. For example, although ARBs vary in their binding affinities in *in vitro* experiments, the significance of the different binding affinities of the various ARBs has rarely been discussed in *in vivo* studies.^{4,5} In *in vitro* experiments that studied the angiotensin II time-dependent dissociation of telmisartan, olmesartan, candesartan, Exp3174 (an active metabolite of losartan) and valsartan from membrane components containing human AT₁ receptors, the dissociation rate constant of each ARB was 0.003248, 0.004171, 0.005203, 0.008561 and 0.009946 min⁻¹, respectively, with corresponding

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half-lives of 213, 166, 133, 81 and 70 min, respectively.⁴ In another study assessing binding to cultured Chinese hamster ovary cells expressing human AT₁ receptors, the dissociation rate constant of olmesartan and telmisartan was 0.0096 and 0.0237 min⁻¹, respectively, with corresponding half-lives of 65 and 29 min, respectively.⁵ Miura *et al.*⁶ reported structural differences among ARBs, and these differences may explain why olmesartan can block AT₁ receptors to a greater degree than other ARBs. However, it is unclear whether the different binding affinities to the AT₁ receptor are reflected in the differences in protection against vascular dysfunction in *in vivo* experiments.

In this study, we used stroke-prone spontaneously hypertensive rats (SHR-SP) that express significantly more of an NADPH oxidase subunit, p22^{phox}, compared with Wistar-Kyoto (WKY) rats.^{7,8} Angiotensin II is well known to induce NADPH oxidase, which in turn has a crucial role in the pathogenesis of vascular dysfunction.^{9–11} Using SHR-SP, olmesartan, a high-affinity ARB and valsartan, a low-affinity ARB, were compared in terms of protection against vascular dysfunction, at doses that showed the same hypotensive effects.

METHODS

Animals

Twelve-week-old male WKY rats and SHR-SP were obtained from Japan SLC (Shizuoka, Japan). The experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (Animal Research Laboratory, Osaka Medical College, Osaka, Japan).

Twelve-week-old SHR-SP received an oral placebo (*n*=6), 3 mg kg⁻¹ valsartan (*n*=6) or 1 mg kg⁻¹ olmesartan (*n*=6) daily for 2 weeks. Systolic blood pressure (SBP) was monitored by tail-cuff plethysmography (BP-98, Softron, Tokyo, Japan). WKY rats were used as a control. After placebo or ARBs were administered for 2 weeks, the rats were weighed and then anesthetized with 35 mg kg⁻¹ of sodium pentobarbital i.p. to obtain blood and tissues. In another experiment, 12-week-old SHR-SP were fed 8% salt, placebo (*n*=20), and valsartan (3 mg kg⁻¹ per day *n*=20) or olmesartan (1 mg kg⁻¹ per day, *n*=20) were administered until a survival rate of 60% was reached.

Renin activity, ACE activity and angiotensin II concentration

Plasma was separated from the blood samples by centrifugation at 3000 r.p.m. for 15 min at 4 °C. Plasma renin activity was determined using an SRL renin kit (TFB, Tokyo, Japan). Vascular tissues were minced and homogenized in 5 vol (wv⁻¹) of 20 mmol l⁻¹ Tris-HCl buffer, pH 8.3 containing 5 mmol l⁻¹ Mg(CH₃COO)₂, 30 mmol l⁻¹ KCl, 250 mmol l⁻¹ sucrose and 0.5% Nonidet P-40.⁷ Vascular and plasma ACE activities were measured using the synthetic substrate, hippil-His-Leu, which was specifically designed to detect ACE (Peptide Institute, Osaka, Japan).⁷ Serum angiotensin II concentrations were measured using an enzyme immunoassay kit (Peninsula Laboratories, Belmont, CA, USA).

Acetylcholine-induced vascular relaxation in isolated rat artery

Isolated rat carotid arteries were cut into 10×1.0 mm helical strips and placed on a myograph under a resting tension of 1.0 g in Tyrode's solution (137 mmol l⁻¹ NaCl, 2.7 mmol l⁻¹ KCl, 1.8 mmol l⁻¹ CaCl₂, 1.1 mmol l⁻¹ MgCl₂, 0.42 mmol l⁻¹ NaH₂PO₄, 12 mmol l⁻¹ NaHCO₃ and 5.7 mmol l⁻¹

glucose, pH 7.4; bathing medium) at 37 °C and then bubbled continuously with 5% CO₂ in O₂.⁷ The strips were initially vasoconstricted with 50 mmol l⁻¹ KCl, and then the bathing medium was washed out. Relaxation induced by acetylcholine (10 μmol l⁻¹) was assessed after contraction to a steady-state tension using 1 μmol l⁻¹ noradrenaline.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA (5 μg) was extracted from aortas using Trizol (Life Technologies, Rockville, MD, USA), in water treated with 0.1% diethyl pyrocarbonate and reverse transcribed using SuperScript II reverse transcriptase and oligo(dT)12–18 primer (Invitrogen, Carlsbad, CA, USA). The reaction proceeded in first-strand buffer, 1 mmol l⁻¹ dNTPs and 20 mmol l⁻¹ dithiothreitol at 42 °C for 50 min. The PCR mixture contained 1 μl of the cDNA reaction, 20 pmol l⁻¹ primers, PCR buffer, 0.4 mmol l⁻¹ dNTPs and 2.5 U Taq polymerase. The reaction was performed in a RoboCycler (Stratagene, La Jolla, CA, USA). The sequences of the PCR primers for p22^{phox} amplification were: sense, 5'-GCTCATCTGTCTGCTGAGTA-3' and antisense, 5'-ACGACCTCA TCTGTCACTGGA-3'; AT₁ receptor: sense, 5'-gcacactggcaatgtaatgc-3' and antisense, 5'-gttgaacagaacaagtacc-3'; and for β-actin: sense, 5'-CCAAGGCC AACCGCGAGAAGATGAC-3' and antisense, 5'-AGGGTACATGGTGGTGC CGCCAGAC-3'. We used β-actin to calibrate sample loading. PCR products were separated by electrophoresis on 2% agarose gels that were subsequently stained with ethidium bromide and visualized using ultraviolet transillumination.⁸

Immunohistochemistry

Fixed aortic tissues were embedded in paraffin and then each block was sectioned at a thickness of 3 μm. The sections were incubated first with 3% H₂O₂ in methanol to inhibit endogenous peroxidase and then with protein-blocking solution (Dako, Tokyo, Japan) to block nonspecific antigens. Sections were incubated with rabbit anti-angiotensin II antibody (IgG, Nashville, TN, USA), goat anti-p22^{phox} antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti-4-hydroxy-2-nonenal (4-HNE) antibody (Nikken Seil, Shizuoka, Japan).¹² A secondary biotinylated anti-IgG antibody (Invitrogen) was used. Sections were visualized using horseradish peroxidase and 3-amino-9-ethylcarbazole as the substrate chromogen (Dako). The nuclei were counterstained using hematoxylin. The immunostain-positive area was quantified with an image analysis system (model VM-30, Olympus Optical, Tokyo, Japan).

Statistical methods

Data are expressed as means ± s.e.m. Statistical analyses were performed using a parametric test with Fisher's Protected Least Significant Difference. Cumulative survival was analyzed for differences according to Kaplan-Meier followed by the log-rank test. Values of *P* < 0.05 were considered to indicate statistical significance.

RESULTS

Blood pressure in SHR-SP

In SHR-SP, SBP before administration of placebo, valsartan and olmesartan was 215 ± 4.2 mm Hg, 215 ± 4.5 mm Hg and 215 ± 4.6 mm Hg, respectively, and in the age-matched WKY rats, SBP was 113 ± 3.1 mm Hg. SBP was 220 ± 3.9 mm Hg after the last dose of placebo, but was decreased similarly by valsartan and olmesartan (Table 1).

Table 1 Effects of valsartan and olmesartan on blood pressure in SHR-SP and salt-loaded SHR-SP

Treatments	WKY rates (<i>n</i> =6)	SHR-SP			Salt-loaded SHR-SP		
		Placebo (<i>n</i> =6)	Valsartan (<i>n</i> =6)	Olmesartan (<i>n</i> =6)	Placebo (<i>n</i> =12)	Valsartan (<i>n</i> =19)	Olmesartan (<i>n</i> =12)
Before treatment (mm Hg)	113 ± 3.1	215 ± 4.2	215 ± 4.5	215 ± 4.6	214 ± 2.3	231 ± 2.4	213 ± 2.4
After last treatment (mm Hg)	114 ± 3.1	220 ± 3.9	213 ± 4.3	212 ± 5.5	265 ± 8.1	247 ± 7.3	259 ± 6.4

Abbreviations: ACE, Angiotensin-converting enzyme; PRA, Plasma renin activity; SHR-SP, stroke-prone spontaneously hypertensive rats; WKY, Wistar-Kyoto. Values are mean ± s.e.m.

Body weight and heart weight in SHR-SP

Body weight was 348 ± 3.6 g in 14-week-old WKY rats and 288 ± 10.1 , 297 ± 3.7 and 299 ± 5.2 g in age-matched placebo-, valsartan- and olmesartan-treated SHR-SP, respectively. There were significant differences between the WKY rats and each of the SHR-SP groups, but no difference among the SHR-SP groups.

Heart weight was 0.92 ± 0.01 g in 14-week-old WKY rats and 1.24 ± 0.02 , 1.24 ± 0.02 and 1.14 ± 0.01 g in age-matched placebo-, valsartan- and olmesartan-treated SHR-SP, respectively. There were significant differences between the WKY rats and each of the SHR-SP groups. However, heart weight in the olmesartan-treated SHR-SP was significantly lower than that in the placebo- or valsartan-treated SHR-SP; the latter two groups had similar heart weight.

The ratios of heart weight to body weight were 2.26 ± 0.03 , 4.32 ± 0.16 , 4.23 ± 0.12 and 3.82 ± 0.09 mg/g in WKY rats, placebo-, valsartan- and olmesartan-treated SHR-SP, respectively. Heart weight to body weight ratio in the WKY rats was significantly lower than in the other groups, but, as with heart weight, heart weight to body weight ratio in the olmesartan-treated SHR-SP was significantly lower than that in the placebo- or valsartan-treated SHR-SP. No significant difference in heart weight to body weight ratio was observed between the placebo- and valsartan-treated SHR-SP.

Plasma renin activity, ACE activity and angiotensin II concentration in SHR-SP

In SHR-SP, renin activity, ACE activity and angiotensin II concentration in plasma and vascular ACE activity after the last treatment are shown in Table 2. Plasma renin activity was similar among the WKY rats and placebo-treated SHR-SP, but was significantly higher in both the valsartan- and the olmesartan-treated groups than in the placebo-treated SHR-SP. Although plasma ACE activity in WKY rats was significantly higher than in each of the SHR-SP groups, there were no significant differences among the SHR-SP groups. Serum angiotensin II concentration was similar in the WKY rats and placebo-treated SHR-SP, but was significantly higher in both the valsartan- and the olmesartan-treated groups than in the placebo-treated SHR-SP. Vascular ACE activity was significantly higher in all the SHR-SP groups than in the WKY group, and there were no significant differences among the SHR-SP groups.

Vascular responses in SHR-SP

In all rats, acetylcholine-induced vascular relaxation was observed as an index of endothelial function (Figure 1). Vascular relaxation was significantly lower in the placebo-treated SHR-SP than in the WKY rats (Figure 1) and was significantly greater in both the valsartan- and the olmesartan-treated SHR-SP than in the placebo-treated SHR-SP. It is noted that vascular relaxation in the olmesartan-treated SHR-SP was significantly greater than that in the valsartan-treated SHR-SP (Figure 1).

Expressions of AT₁ receptor and p22^{phox} in SHR-SP

AT₁ receptor expression was similar in all four groups (Figure 2). On the other hand, an NADPH oxidase subunit p22^{phox} expression increased significantly in the placebo-treated SHR-SP when compared

Figure 1 Acetylcholine-induced vascular relaxation in noradrenaline-precontracted carotid arteries in WKY rats, placebo-, valsartan (Val)- and olmesartan (Olm)-treated SHR-SP. ** $P < 0.01$ vs. placebo-treated SHR-SP. † $P < 0.05$ vs. valsartan-treated SHR-SP.

Figure 2 Expressions of (a) AT₁ receptor and (b) p22^{phox} in aorta obtained from WKY rats, placebo (P)-, valsartan (V)- and olmesartan (O)-treated SHR-SP. * $P < 0.05$ and ** $P < 0.01$ vs. placebo-treated SHR-SP. † $P < 0.05$ vs. valsartan-treated SHR-SP.

Table 2 Effects of valsartan and olmesartan on PRA, plasma and vascular ACE activities, serum angiotensin II concentration in SHR-SP

Parameters	WKY rates (n=6)	SHR-SP		
		Placebo (n=6)	Valsartan (n=6)	Olmesartan (n=6)
PRA (ng AI ml ⁻¹ per h)	6.6 ± 1.2	6.1 ± 1.0	$13.1 \pm 1.1^{**}$	$14.3 \pm 1.8^{**}$
Plasma ACE activity (mU ml ⁻¹)	$98.0 \pm 3.8^{**}$	46.5 ± 2.1	43.9 ± 2.2	44.2 ± 2.4
Serum angiotensin II concentration (pg ml ⁻¹)	17.7 ± 1.3	16.1 ± 2.8	$33.7 \pm 4.1^{**}$	$34.7 \pm 3.0^{**}$
Vascular ACE activity (mU mg ⁻¹ protein)	$3.14 \pm 0.2^{**}$	4.33 ± 0.21	4.22 ± 0.2	3.95 ± 0.3

Abbreviations: ACE, Angiotensin-converting enzyme; PRA, Plasma renin activity; SHR-SP, stroke-prone spontaneously hypertensive rats; WKY, Wistar-Kyoto. Values are mean $\pm$ s.e.m. ** $P < 0.01$ vs. placebo.

with the WKY rats. However, p22^{phox} expression was significantly lower in both the valsartan- and the olmesartan-treated SHR-SP than in the placebo-treated SHR-SP (Figure 2). Nevertheless, p22^{phox} expression in the olmesartan-treated SHR-SP was significantly lower than in the valsartan-treated SHR-SP (Figure 2).

Immunohistochemistry in SHR-SP

Figure 3 shows the angiotensin II-positive area in the aortas, reflecting the level of angiotensin II bound to AT₁ receptors. There were few angiotensin II-positive areas in the medial lesions of the aortas of WKY rats, but such areas were large in these lesions in the placebo-treated SHR-SP (Figure 3). On the other hand, the angiotensin II-positive areas in the medial lesions of both the valsartan- and the olmesartan-treated SHR-SP were smaller than those in the placebo-treated SHR-SP (Figure 3). The ratios of angiotensin II-positive area to medial area were significantly lower in both the valsartan- and the olmesartan-treated SHR-SP than in the placebo-treated SHR-SP, and this ratio was significantly lower in the olmesartan-treated SHR-SP than in the valsartan-treated SHR-SP (Figure 3).

The vascular p22^{phox}-positive area was obviously larger in the placebo-treated SHR-SP than in the WKY rats, but in both the valsartan- and the olmesartan-treated SHR-SP, the areas were smaller than in the placebo-treated SHR-SP (Figure 4). Significant attenuations of the ratios of p22^{phox}-positive area to medial area were observed in both the valsartan- and the olmesartan-treated SHR-SP when compared with the placebo-treated SHR-SP, and a significant attenuation of the ratio in the olmesartan-treated SHR-SP was also observed when compared with the valsartan-treated SHR (Figure 4).

To evaluate the products of oxidative stress, we evaluated the 4-HNE-positive area in the aortas. This area was small in the WKY rats, but was much larger in the placebo-treated SHR-SP. On the other hand, although the 4-HNE-positive areas were observed in the

ARB-treated groups, they were smaller than those in the placebo-treated group (Figure 5). The ratios of the 4-HNE-positive area to medial area were significantly smaller in the olmesartan-treated SHR-SP than in the valsartan-treated SHR-SP (Figure 5).

Cumulative survival in salt-loaded SHR-SP

Systolic blood pressures were 214 ± 2.3 , 213 ± 2.4 and 213 ± 2.4 mm Hg, respectively, in the placebo-, valsartan- and olmesartan-treated groups before salt loading (Table 1). The placebo-treated SHR-SP began to die at 11 days after salt loading, and the survival rate reached 60% at 25 days (Figure 6). Postmortem macroscopic examination of the brain revealed that all animals had cerebral hemorrhage and/or infarction. At the end of the experiment, systolic blood pressure in the surviving placebo-treated group was 265 ± 8.1 mm Hg, and there were no significant differences among the three groups (Table 1). On the other hand, the valsartan-treated SHR-SP began to die at 14 days after salt loading, and the survival rate reached 60% at 42 days (Figure 6). There was a significant difference in mortality between the placebo and valsartan groups. The olmesartan-treated SHR-SP did not die until 34 days, in the 42-day observation period, and only one rat died at 35 days (Figure 6). Significant differences in mortality were observed between the placebo and olmesartan groups and between the olmesartan and valsartan groups.

Vascular response, p22^{phox} expression and 4-HNE-positive area in salt-loaded SHR-SP

The ratio of vascular relaxation induced by acetylcholine to that by papaverine in the placebo-treated SHR-SP after salt loading was $55.6 \pm 2.9\%$ (Figure 7). The ratio in the olmesartan-treated SHR-SP after salt loading was significantly greater than that in the placebo- or valsartan-treated SHR-SP, and there was no significant difference between the placebo- or valsartan-treated SHR-SP (Figure 7).

Figure 3 (a) Typical photographs of anti-angiotensin II antibody-stained aortas in WKY rats, placebo-, valsartan- and olmesartan-treated SHR-SP. (b) Ratio of anti-angiotensin II antibody-stained area to medial area in WKY rats, placebo-, valsartan- and olmesartan-treated SHR-SP. ** $P < 0.01$ vs. placebo-treated SHR-SP. †† $P < 0.01$ vs. valsartan-treated SHR-SP.

Figure 4 (a) Typical photographs of anti-p22^{phox} antibody-stained aortas in WKY rats, placebo-, valsartan- and olmesartan-treated SHR-SP. (b) Ratio of anti-p22^{phox} antibody-stained area to medial area in WKY rats, placebo-, valsartan- and olmesartan-treated SHR-SP. ** $P < 0.01$ vs. placebo-treated SHR-SP. † $P < 0.05$ vs. valsartan-treated SHR-SP.

Figure 5 (a) Typical photographs of the anti-4-HNE antibody-stained aortas in WKY rats, placebo-, valsartan- and olmesartan-treated SHR-SP. (b) Ratio of anti-4-HNE antibody-stained area to medial area in WKY rats, placebo-, valsartan- and olmesartan-treated SHR-SP. ** $P < 0.01$ vs. placebo-treated SHR-SP. †† $P < 0.01$ vs. valsartan-treated SHR-SP.

Figure 6 Cumulative percent survival in placebo-, valsartan- and olmesartan-treated SHR-SP fed 8% salt. The differences in percent survival between placebo- and valsartan-treated SHR-SP and between placebo and olmesartan groups were statistically significant (Kaplan–Meier analysis followed by log rank test; $P < 0.05$, placebo vs. valsartan; $P < 0.01$, placebo vs. olmesartan). The difference in percent survival between the valsartan and olmesartan groups was statistically significant (Kaplan–Meier analysis followed by log rank test; $P < 0.05$, valsartan vs. olmesartan).

The vascular expression of p22^{phox} was significantly lower in the olmesartan-treated SHR-SP after salt loading than in the placebo- and valsartan-treated SHR-SP (Figure 7). However, no significant difference in p22^{phox} expression was observed between the placebo- or valsartan-treated SHR-SP (Figure 7).

The ratios of the 4-HNE-positive area to medial area were significantly smaller in the olmesartan-treated SHR-SP after salt loading than in the placebo- or valsartan-treated SHR-SP, and there was no significant difference between the placebo- or valsartan-treated SHR-SP (Figure 7).

Vascular ACE activity and AT₁ receptor expression in salt-loaded SHR-SP

After salt loading, vascular ACE activity was significantly lower in the olmesartan-treated SHR-SP than in the placebo- or valsartan-treated rats, and there was no significant difference between the placebo- or valsartan-treated SHR-SP (Figure 8).

Vascular AT₁ receptor expression tended to be lower in the olmesartan-treated SHR-SP after salt loading than in the placebo- or valsartan-treated rats, but no significant differences were observed among the three groups (Figure 8).

DISCUSSION

In this study, we showed that the angiotensin II-positive area in the aortic medial lesions was significantly greater in placebo-treated SHR-SP than in WKY rats. This area indicates the level of angiotensin II bound to AT₁ receptors. This study concurs with previous research showing that vascular ACE activity was significantly increased in SHR-SP compared with WKY rats, indicating the upregulation of angiotensin II formation.^{7,8} However, the expression of AT₁ receptors did not differ between WKY rats and SHR-SP. Therefore, the increase in the angiotensin II-positive area might depend on the upregulation of angiotensin II formation, but not on the upregulation of AT₁ receptor expression. The size of the angiotensin II-positive area was significantly reduced by both olmesartan and valsartan. However, the degree of attenuation was significantly greater in the olmesartan treatment

Figure 7 (a) Acetylcholine-induced vascular relaxation in noradrenaline-precontracted carotid arteries in placebo (P)-, and valsartan (V)-, and olmesartan (O)-treated SHR-SP fed 8% salt. (b) Expressions of p22^{phox} in aorta obtained from placebo (P)-, valsartan (V)- and olmesartan (O)-treated SHR-SP fed 8% salt. (c) Ratio of anti-4-HNE antibody-stained area to medial area in placebo (P)-, valsartan (V)- and olmesartan (O)-treated SHR-SP fed 8% salt. ** $P < 0.01$ vs. placebo-treated SHR-SP fed 8% salt. †† $P < 0.01$ vs. valsartan-treated SHR-SP fed 8% salt.

than in the valsartan treatment for doses that produced the same hypotensive effects. In *in vitro* experiments, the affinity of olmesartan for AT₁ receptors is clearly higher than that of valsartan.⁴ In this study, a significant reduction of the angiotensin II-positive area was observed in SHR-SP after olmesartan treatment when compared with valsartan treatment. These findings suggest that high affinity ARB may be more effective than low affinity ARB, *in vivo* as well as *in vitro*.

Angiotensin II has been reported to stimulate NADPH oxidase, thereby inducing oxidative stress, although the signaling mechanisms involved are not fully understood. In cultured vascular smooth muscle cells, these signals may include protein kinase C, tyrosine kinases, phosphatidylinositol-3-kinase and Rac (a small molecular weight G-protein).⁹ Expression of the NADPH oxidase subunit p22^{phox} in vascular tissues was evaluated in this study. Elevated p22^{phox}

Figure 8 (a) ACE activity in aorta obtained from placebo (P)-, valsartan (V)- and olmesartan (O)-treated SHR-SP fed 8% salt. (b) Expressions of AT₁ receptor in aorta obtained from placebo (P)-, valsartan (V)- and olmesartan (O)-treated SHR-SP fed 8% salt. ** $P < 0.01$ vs. placebo-treated SHR-SP fed 8% salt. †† $P < 0.01$ vs. valsartan-treated SHR-SP fed 8% salt.

expression reflects an increase in NADPH oxidase activity, which is well known to be closely related to oxidative stress caused by angiotensin II in the vascular tissue of hypertensive rats, and the oxidative stress caused by NADPH oxidase has an important role in the development and progression of vascular remodeling.^{9–11} Expression of p22^{phox} in vascular tissues was significantly higher in the placebo-treated SHR-SP than in the WKY rats in this study, as has also been reported earlier.^{7,8} In the histological evaluation, not only the angiotensin II-positive area but also the p22^{phox}-positive area was significantly augmented in the placebo-treated SHR-SP compared with the WKY rats. Furthermore, an oxidative stress marker 4-HNE was also higher in the SHR-SP than in the WKY rats. These findings clearly suggest that an increase of angiotensin II stimulation augmented p22^{phox} expression that further induced oxidative stress in the vascular tissues. Liu *et al.*¹³ reported that an inhibitor of the NADPH oxidase subunit gp91^{phox} attenuated vascular hypertrophy induced by angiotensin II, without a reduction of blood pressure. Jinno *et al.*¹⁴ reported that neither 0.5 mg kg⁻¹ nor 3 mg kg⁻¹ of olmesartan affected BP in normotensive mice, but that 3 mg kg⁻¹ but not 0.5 mg kg⁻¹ olmesartan significantly reduced p22^{phox} expression. Therefore, the attenuation of p22^{phox} expression might not necessarily depend on the hypotensive effects of ARB. In this study, the angiotensin II-, p22^{phox}- and 4-HNE-positive areas were significantly smaller in the olmesartan-treated SHR-SP than in the valsartan-treated SHR-SP despite using doses with the same hypotensive effect. These findings might indicate that the greater blockade of AT₁ receptors by olmesartan resulted in more powerful attenuation of oxidative stress in the vascular tissues.

Acetylcholine-induced vascular relaxation, which is used as an index of endothelial function, was significantly lower in SHR-SP than in WKY rats, as reported earlier.^{7,8,15} Endothelial function is directly affected by nitric oxide bioavailability, and its dysfunction is associated with oxidative stress induced by angiotensin II.^{16,17} In an earlier report, although losartan and trichlormethiazide at equivalent hypotensive doses were administered for 2 weeks in SHR-SP, the mRNA levels of NADPH oxidase subunits and oxidative stress were significantly reduced by losartan, but not by trichlormethiazide.¹⁸ Pu *et al.*¹⁹ showed that valsartan and hydralazine showed the same hypotensive effect in SHR-SP, but that valsartan, but not hydralazine, reduced endothelial dysfunction. Therefore, angiotensin II receptor blockade may be more useful for preventing endothelial function than

for its hypotensive effect. In this study, the acetylcholine-induced vascular relaxation was significantly greater in both the olmesartan- and the valsartan-treated SHR-SP than in the placebo-treated SHR-SP. However, the degree of relaxation was significantly greater in olmesartan-treated SHR-SP than in valsartan-treated SHR-SP, although there was no significant difference in SBP between these two ARBs. The endothelial dysfunction may be related to oxidative stress in the vascular tissues. In fact, attenuation of angiotensin II-, p22^{phox}- and the 4-HNE-positive areas was significantly stronger for olmesartan than for valsartan. Thus, the protective effect of endothelial dysfunction by ARBs may also depend on the degree of angiotensin II blockade in vascular tissues.

We further studied whether lifespan was augmented by olmesartan and valsartan, and whether lifespan extension differed between olmesartan and valsartan. Earlier, we studied whether the ACE inhibitor, trandolapril, extended the survival rate in the SHR-SP fed 8% salt, that is, the same model as in this study.³ This salt-loaded SHR-SP is a model of very severe hypertension in which SBP exceeds 250 mm Hg even 2 weeks after salt loading and the survival rate reduces to 15% at 4 weeks.³ In that study, although trandolapril did not lower SBP after salt loading, the lifespan of trandolapril-treated rats was significantly augmented compared with that in placebo-treated rats. In this study, the rate of survival reached 60% at 25 days after salt loading, and at that point SBP exceeded 250 mm Hg. Although neither olmesartan nor valsartan affected SBP, they significantly extended the time to reach the survival rate of 60% compared with placebo. However, olmesartan-treated rats had a survival rate of 95% at 42 days when the valsartan-treated rats reached the survival rate of 60%. The surviving rats treated with olmesartan, but not those treated with valsartan, showed significant attenuation of acetylcholine-induced vascular relaxation and significantly reduced vascular p22^{phox} expression. The vascular ACE activity did not differ significantly between olmesartan and valsartan in SHR-SP (Table 2), but the activity was significantly attenuated by treatment with olmesartan, but not with valsartan, in the salt-loaded SHR-SP (Figure 8). Although both valsartan and olmesartan prevented the endothelial dysfunction in SHR-SP, only olmesartan had this protective effect in salt-loaded SHR-SP. Valsartan's protective effect against endothelial dysfunction might have disappeared in salt-loaded SHR-SP because valsartan, unlike olmesartan, could not inhibit the increase of vascular ACE activity after salt loading. Earlier reports showed that vascular ACE activity was increased after salt loading in SHR-SP, and the same phenomenon was also observed in this study.^{3,7} The increased ACE activity might augment angiotensin II formation and induce additional oxidative stress, resulting in further impairment of endothelial function. Valsartan could not inhibit the increased ACE activity after salt loading, and this might account for the absence of the protective effect that was observed in SHR-SP. In earlier experimental models, olmesartan reduced vascular ACE activity.^{20,21} However, in this experiment using SHR-SP, olmesartan could not reduce vascular ACE activity. This discrepancy between the earlier reports and our study may reflect the difference in experimental periods (2 weeks in our study vs. over 4 weeks in earlier reports). On the other hand, in the salt-loaded SHR-SP, olmesartan could attenuate the vascular ACE activity. Recently, Agata *et al.*²¹ showed that olmesartan increased angiotensin-(1-7) through ACE2 expression. Angiotensin-(1-7) is known to inhibit the ACE C-domain, resulting in acting as an ACE inhibitor.²² Although the mechanism of olmesartan to inhibit ACE activity remains unclear, olmesartan's effect of lifespan extension may involve the attenuation of vascular ACE activity, in addition to the direct AT₁ receptor blockade in this study.

We did not measure SBP in the salt-loaded SHR-SP during the experiment. The salt-loaded SHR-SP is known to be a model of severe hypertensives. In our earlier studies, an ACE inhibitor, trandolapril and the ARBs telmisartan and valsartan, at doses that showed significant hypotensive effects in SHR-SP, could not lower the blood pressure at 2 weeks after sodium loading.^{3,7} The doses of both olmesartan and valsartan used in this study did not lower the blood pressure significantly even in SHR-SP; hence, it is possible that ARB did not affect the blood pressure after sodium loading. However, both olmesartan and valsartan extended the lifespan compared with placebo in the sodium-loaded SHR-SP. Therefore, these ARBs may have an effect that extends the lifespan beyond lowering the blood pressure. On the other hand, the superior effect of olmesartan compared with valsartan was observed in the protection of the endothelial function and the extension of lifespan in the sodium-loaded SHR-SP. Whether the superior effect is adapted to the clinical setting is not clear, and a clinical study may be needed as a future study.

In conclusion, olmesartan, an ARB with high affinity to AT₁ receptors, showed better vascular protection than valsartan, a low-affinity ARB, despite using doses with the same hypotensive effect. Such heterogeneity in binding affinity to AT₁ receptors among ARBs may result in varying vascular protection and lifespan extension.

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