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Antihypertensive, insulin-sensitising and renoprotective effects of a novel, potent and long-acting angiotensin II type 1 receptor blocker, azilsartan medoxomil, in rat and dog models

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ABSTRACT

The pharmacological profile of a novel angiotensin II type 1 receptor blocker, azilsartan medoxomil, was compared with that of the potent angiotensin II receptor blocker olmesartan medoxomil. Azilsartan, the active metabolite of azilsartan medoxomil, inhibited the binding of [^{125}I]–Sar^{1}–Ile^{8}-angiotensin II to angiotensin II type 1 receptors. Azilsartan medoxomil inhibited angiotensin II-induced pressor responses in rats, and its inhibitory effects lasted 24 h after oral administration. The inhibitory effects of olmesartan medoxomil disappeared within 24 h. ID_{50} values were 0.12 and 0.55 mg/kg for azilsartan medoxomil and olmesartan medoxomil, respectively. In conscious spontaneously hypertensive rats (SHRs), oral administration of 0.1–1 mg/kg azilsartan medoxomil significantly reduced blood pressure at all doses even 24 h after dosing. Oral administration of 0.1–3 mg/kg olmesartan medoxomil also reduced blood pressure; however, only the two highest doses significantly reduced blood pressure 24 h after dosing. ED_{25} values were 0.41 and 1.3 mg/kg for azilsartan medoxomil and olmesartan medoxomil, respectively. In renal hypertensive dogs, oral administration of 0.1–1 mg/kg azilsartan medoxomil reduced blood pressure more potently and persistently than that of 0.3–3 mg/kg olmesartan medoxomil. In a 2-week study in SHRs, azilsartan medoxomil showed more stable antihypertensive effects than olmesartan medoxomil and improved the glucose infusion rate, an indicator of insulin sensitivity, more potently (≥10 times) than olmesartan medoxomil. Azilsartan medoxomil also exerted more potent antiproteinuric effects than olmesartan medoxomil in Wistar fatty rats. These results suggest that azilsartan medoxomil is a potent angiotensin II receptor blocker that has an attractive pharmacological profile as an antihypertensive agent.

1. Introduction

In 2000, 972 million adults worldwide were diagnosed with hypertension and this number is predicted to increase by approximately 60% (to 1.56 billion) by 2025 (Kearney et al., 2005). Several clinical trials have indicated that strict blood pressure control is a major therapeutic strategy for reducing cardiovascular and renal morbidities and mortalities (Chobanian et al., 2003; Elliott, 2004; Vasan et al., 2002). However, even with current antihypertensive management strategies, only one-third of hypertensive patients achieve their goals for systolic (<140 mm Hg) and diastolic (<90 mm Hg) blood pressures (Chobanian et al., 2003; Ferrario et al., 2004).

To address this issue, the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure, the European Society of Hypertension-European Society of Cardiology Task Force on the Management of Arterial Hypertension and the Japanese Society of Hypertension Committee have proposed an aggressive approach for hypertension management that utilises combination therapy (Chobanian et al., 2003; Mancia et al., 2007; Ogihara et al., 2009). Possible options for such a therapeutic model would include use of well-tolerated agents that could significantly reduce blood pressure compared with other drugs of the same class in addition to antihypertensive drugs with new action mechanisms.

The clinical efficacies of angiotensin II type 1 (angiotensin AT_1) receptor blockers, such as losartan, candesartan cilexetil, valsartan,

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irbesartan, telmisartan and olmesartan medoxomil, have been established in hypertensive patients (Easthope and Jarvis, 2002; Graettinger, 2003; Stumpe, 2004; Weber, 2002). Because angiotensin II receptor blockers are generally considered to be better tolerated than other classes of antihypertensive drugs, they are preferentially used in both monotherapy and combination therapy for essential hypertension. Among the currently available angiotensin II receptor blockers, olmesartan medoxomil is the newest to the market and has been reported to be the best in its class in terms of blood pressure reduction (Stumpe, 2004). However, the need for compounds with improved antihypertensive efficacy remains.

With this aim, we developed a new antihypertensive agent, azilsartan medoxomil [TAK-491; (5-methyl-2-oxo-1,3-dioxol-4-yl) methyl 2-ethoxy-1-{[2′-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl) biphenyl-4-yl]methyl}-1*H*-benzimidazole-7-carboxylate monopotassium salt] (Fig. 1). Azilsartan medoxomil is a prodrug that is rapidly hydrolysed to the active moiety azilsartan, which is a potent and selective antagonist of the angiotensin AT_1 receptor. In comparison with other angiotensin II receptor blockers, azilsartan slowly dissociates from the receptor and persistently inhibits the angiotensin II-induced accumulation of inositol 1,4,5-trisphosphate even after washout in Chinese hamster ovary (CHO) cells which overexpress the human angiotensin AT_1 receptor (Ojima et al., 2011). The purpose of the present study was to characterise the antihypertensive, insulin-sensitising and antiproteinuric effects of azilsartan medoxomil in rat and dog models, and to compare these effects with those of olmesartan medoxomil.

2. Materials and methods

All animal experiments were performed according to the guidelines of the Takeda Experimental Animal Care and Use Committee.

2.1. Drugs and materials

Azilsartan [TAK-536; 2-ethoxy-1-{[2′-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl)biphenyl-4-yl]methyl}-1*H*-benzimidazole-7-carboxylic acid] and azilsartan medoxomil [TAK-491; (5-methyl-2-oxo-1,3-dioxol-4-yl)methyl 2-ethoxy-1-{[2′-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl)biphenyl-4-yl]methyl}-1*H*-benzimidazole-7-carboxylate monopotassium salt] were synthesised at Chemical Development Laboratories, Takeda Pharmaceutical Company Ltd. (Osaka, Japan). Olmesartan medoxomil was obtained from KNC Laboratories Co. Ltd. (Kobe, Japan). Olmesartan was purified by Takeda Pharmaceutical Company Ltd. Azilsartan medoxomil and olmesartan medoxomil were suspended in 0.5% w/v methylcellulose solution and orally administered at a volume of 2 ml/kg. The vehicle control groups were administered 0.5% w/v methylcellulose.

2.2. Binding assay for human angiotensin receptors

Ninety-six-well FlashPlates® into which membranes purified from human angiotensin AT_1 receptor-expressing CHO cells were immobilised (hAT_1 angiotensin II ScreenReady™ Targets) and [^{125}I]–Sar^1–Ile^8–angiotensin II (2200 Ci/mmol) were purchased from PerkinElmer (Boston, MA, USA). Angiotensin II was purchased from Peptide Institutes, Inc. (Osaka, Japan). An [^{125}I]–Sar^1–Ile^8–angiotensin II displacement binding assay was performed using human angiotensin AT_1 receptor-coated plates that contained 2.3 fmol of the receptor per well (4.0 μg of the membrane protein per well). The membrane-coated wells were incubated with an assay buffer (50 mmol/l Tris–HCl buffer with 5 mmol/l $MgCl_2$ and 1 mmol/l EDTA; pH 7.4) that contained varying concentrations of azilsartan (final concentration, 0.01–10 nmol/l), olmesartan (final concentration, 0.01–10 nmol/l) or angiotensin II (final concentration, 1–3000 nmol/l). After 90 min, [^{125}I]–Sar^1–Ile^8–angiotensin II (final concentration, 0.6 nmol/l) was added and the membranes were further incubated for 120 min at room temperature. The amount of radioactivity trapped on the membrane was measured using a microplate scintillation and luminescence counter (TopCount C991201; PerkinElmer). Non-specific binding of [^{125}I]–Sar^1–Ile^8–angiotensin II was estimated in the presence of 10 μmol/l unlabeled angiotensin II. Specific binding was calculated by subtracting the amount of non-specific binding from the amount of total binding measured. In addition, an [^{125}I]–CGP–42112A displacement binding assay was performed using human angiotensin AT_2 receptors derived from transfected Hela cells. For the AT_2 receptor binding assay, [^{125}I]–CGP–42112A (final concentration, 0.025 nmol/l) was incubated with the assay buffer for 180 min in the presence or absence of azilsartan (final concentration, 10 μmol/l).

The concentration of compounds required for 50% inhibition (IC_{50}) was determined by non-linear logistic regression analysis using the SAS software (ver.8.2; SAS Institute Japan Ltd., Tokyo, Japan). The reported values represent the mean results of triplicate wells.

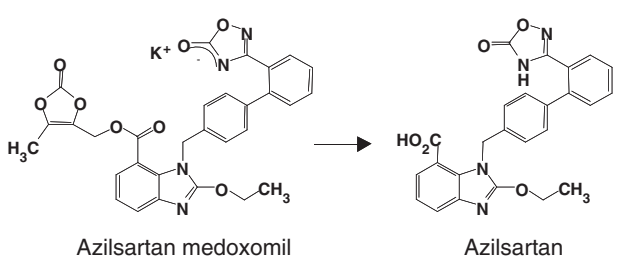

Fig. 1. Chemical structure of azilsartan medoxomil and its active metabolite, i.e. azilsartan.

2.3. Angiotensin II-induced pressor response in rats

Ten-week-old male Sprague Dawley rats (total 40 rats; CLEA Japan, Inc., Tokyo, Japan) were anaesthetised with a 50-mg/kg intraperitoneal injection of sodium pentobarbital. Their femoral arteries and veins were isolated and cannulated using polyethylene catheters filled with saline containing 200 U/ml heparin. The catheters were passed subcutaneously and exteriorised at the back of the neck.

After a recovery period, the arterial catheter was connected to a polygraph system (NEC San-ei Instruments Ltd. and Nihon Kohden Corporation, Tokyo, Japan) that monitored the mean blood pressure. At least two injections of angiotensin II (100 ng/kg each) were administered into the venous catheter to confirm a stable response and to determine any pre-treatment elevations in the mean blood pressure. Rats with an unstable pressor response or a response of <35 mm Hg were excluded from the experiment.

Angiotensin II was injected 1, 3, 5, 7, 10 and 24 h after oral administration of azilsartan medoxomil or olmesartan medoxomil, and increases in the mean blood pressure were measured. The inhibition rate for elevation in the pre-treatment blood pressure was calculated at each measurement time point.

Drug doses that reduced increases in the mean blood pressure by 50% (ID_{50}) were calculated from the area over the curve (AOC) for a 24-h time period ($AOC_{0-24\,h}$; percent inhibition × h) by non-linear logistic regression analysis.

2.4. Antihypertensive effects in spontaneously hypertensive rats (SHRs)

In this study, 38–40-week-old male SHRs (total 58 rats; SHR/lzm, Japan SLC, Inc., Shizuoka, Japan) were used. The rats were anaesthetised by intraperitoneal administration of 50 mg/kg sodium pentobarbital. The left femoral artery was cannulated using a polyethylene catheter filled with saline containing 200 U/ml heparin. The catheter was exteriorised at the back of the neck. Each SHR was individually housed in a cage and left overnight to recover.

The blood pressure was monitored from the femoral artery, and the heart rate was measured from the blood pressure and pulse intervals. Rats with a mean blood pressure of >150 mm Hg were included in the experiment. When the mean blood pressure stabilised, azilsartan medoxomil or olmesartan medoxomil was orally administered and the mean blood pressure was recorded for 24 h. All blood pressure measurements were performed in a blinded manner.

Drug doses that reduced the mean blood pressure by 25 mm Hg (ED_{25}) were calculated from $AOC_{0-24\,h}$ (mm Hg × h) divided by 24 using the linear least squares method.

In the experiment involving repeated drug administrations, 29–31-week-old male SHRs (total 55 rats) were orally administered 0.1, 0.3 or 1 mg/kg azilsartan medoxomil, 1, 3 or 10 mg/kg olmesartan medoxomil or the vehicle once daily for 2 weeks. Using the tail-cuff method, the systolic blood pressure and heart rate were measured (BP-98A; Softron, Tokyo, Japan) before and 5 and 24 h after drug administration on days 1, 7 and 14. To detect any potential rebounding, the systolic blood pressure and heart rate were also measured on days 2, 7 and 14 after the final dose was administered.

2.5. Antihypertensive effects in renal hypertensive dogs

Male beagle dogs (total 5 dogs; age 16–20 months; Kitayama Labs, Nagano, Japan) were anaesthetised by intravenous administration of 30 mg/kg sodium pentobarbital and the left renal artery was isolated. An electromagnetic probe (FB-030 T; Nihon Kohden Corporation) connected to a flow metre (MFV-2100; Nihon Kohden Corporation) was placed around the vessel. Following stabilisation of renal blood flow, the artery was constricted using a silver clip to reduce the blood flow rate to 10–20 ml/min.

Blood pressure measurements were performed 6 and 11 weeks after surgery. The systolic blood pressure and heart rate were indirectly measured from the right forearm (BP-98E; Softron). Measurements were repeated in triplicate and the mean value was used for analyses.

Dogs with a systolic blood pressure of >190 mm Hg were included in the experiment and were orally administered the vehicle, 0.1 or 1 mg/kg azilsartan medoxomil or 0.3 or 3 mg/kg olmesartan medoxomil in a crossover design (a 5 × 5 Latin square was used to allocate the treatments). The systolic blood pressure and heart rate were measured 1, 3, 5, 7, 10 and 24 h after oral administration. Each dog received different treatments that were separated by a washout period of at least 5 days.

$AOC_{0-24\,h}$ (mm Hg × h) was calculated to directly compare the effects of azilsartan medoxomil and olmesartan medoxomil.

2.6. Measurement of insulin sensitivity in SHRs

Male SHRs (total 72 rats; age 24–26 weeks) with equalised body weights, systolic blood pressures and fasting plasma glucose and insulin levels were administered either the vehicle, azilsartan medoxomil or olmesartan medoxomil. The vehicle, 0.1, 0.3 or 1 mg/kg azilsartan medoxomil or 1, 3 or 10 mg/kg olmesartan medoxomil were orally administered once daily for 2 weeks. After the final administration, the rats were fasted overnight and insulin sensitivity was measured using the hyperinsulinaemic–euglycaemic clamp technique (Furuhashi et al., 2002).

In brief, the rats were anaesthetised by intraperitoneal administration of 50 mg/kg sodium pentobarbital. The right common carotid artery was catheterised for blood sampling. Both femoral veins were also catheterised; one was used for insulin infusion and the other for glucose infusion. A bolus injection of 25 mU/kg insulin was intravenously administered, followed by an infused dose of 4 mU/kg/min insulin for 100 min.

The blood glucose level was clamped at a normal value by adjusting the infusion rate of a 50% glucose solution that was delivered via the femoral cannula. This process was guided by measuring blood glucose levels of samples obtained from the common carotid artery at 5-min intervals. The mean glucose infusion rate during the last 40 min of clamping was considered as an indicator of insulin sensitivity. The plasma insulin level that was measured when the insulin infusion was stopped was defined as the steady-state plasma insulin level.

Plasma glucose levels of each group were enzymatically measured using the Hitachi 7070 autoanalyser (Hitachi, Ibaraki, Japan). Plasma insulin levels were measured using a commercially available radioimmunoassay kit (Shionogi Pharmaceutical, Osaka, Japan). In the glucose clamping study, blood glucose levels were measured using a portable glucose analyser (Roche Diagnostics Japan, Tokyo, Japan).

2.7. Antiproteinuric effects in Wistar fatty rats

In this study, 25-week-old male Wistar fatty rats, which are a model of diabetic nephropathy, and age-matched male Wistar lean rats (total 35 rats; Takeda Rabics, Osaka, Japan) were used. Wistar fatty rats with equalised urinary albumin and total protein excretion levels, plasma glucose levels, systolic blood pressures and body weights were divided into five groups. The groups were orally treated with the vehicle, 0.3 or 1 mg/kg azilsartan medoxomil or 3 or 10 mg/kg olmesartan medoxomil. Wistar lean rats were treated with the vehicle and served as normal controls.

The drugs were orally administered to the rats once daily starting at 26 weeks of age. After 4 weeks of treatment, the rats were placed in metabolic cages equipped with drinking bottles and food cups outside the cages, and 24-h urine samples were collected to measure urinary albumin and total protein levels. For biochemical analyses, blood samples were collected from the tail vein using heparin as an anticoagulant.

To measure urinary albumin and total protein levels, urine samples were desalted on a gel filtration column (PD-10; Amersham Biosciences, Uppsala, Sweden) equilibrated with a 0.04% w/v ammonium carbonate solution. Urinary albumin and total protein levels were measured using an assay kit (Wako Pure Chemical Industries, Osaka, Japan). Plasma glucose, triglyceride and total cholesterol levels were enzymatically measured using the Hitachi 7070 autoanalyser. The plasma immunoreactive insulin level was measured using the above-mentioned radioimmunoassay kit.

2.8. Statistical analyses

All data are expressed as the mean ± standard error of the mean (S.E.M.). Drug treatment data were compared with the vehicle treatment data using Williams or Shirley–Williams test followed by Bonferroni correction for time point comparisons or the contrast test based on crossover ANOVA with Bonferroni correction, using the SAS software. Student's *t*-test was used for direct comparisons of the effects of azilsartan medoxomil and olmesartan medoxomil. For experiments involving proteinuria, differences between all the parameters measured in the vehicle-treated Wistar fatty and lean rats were analysed using the Student's *t*-test or Aspin–Welch test. P-values of ≤0.025 in the Williams test, Shirley–Williams test and contrast test and P-values of ≤0.05 in the Student's *t*-test and Aspin–Welch test indicate statistically significant differences.

3. Results

3.1. Inhibition of binding of [^{125}I]–Sar^1–Ile^8–angiotensin II to human angiotensin AT_1 receptors

Azilsartan inhibited the specific binding of [^{125}I]–Sar^1–Ile^8–angiotensin II to human angiotensin AT_1 receptors in a concentration-related manner with an IC_{50} value of 0.62 nmol/l (Fig. 2). Olmesartan

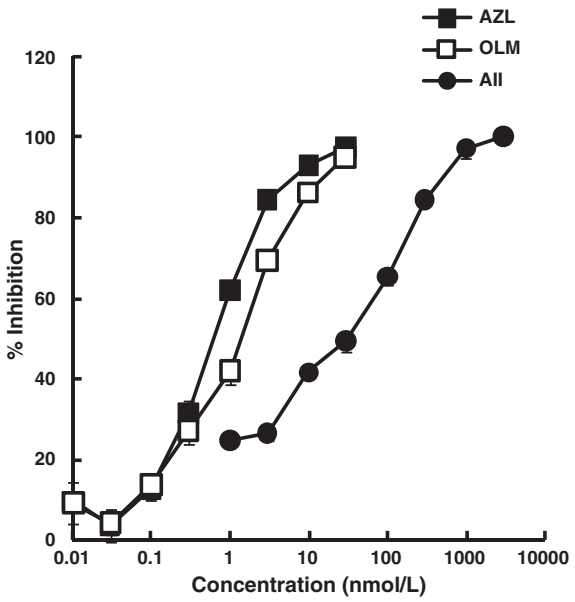

Fig. 2. Displacement of specific binding of [^{125}I]–Sar^{1}–Ile^{8}–angiotensin II (final concentration, 0.6 nmol/l) to human AT_1 receptors by azilsartan (AZL), olmesartan (OLM) and angiotensin II. Values represent the mean of triplicate wells.

and angiotensin II also inhibited this binding with IC_{50} values of 1.2 and 20 nmol/l, respectively (Fig. 2). These results demonstrate that azilsartan and olmesartan bind to angiotensin AT_1 receptors with high affinity. In contrast to the AT_1 receptor, azilsartan inhibited the binding of [^{125}I]–CGP–42112A to the human angiotensin AT_2 receptor only by 2% at a concentration of 10 μmol/l, indicating that azilsartan is a selective angiotensin AT_1 receptor blocker.

3.2. Inhibition of the angiotensin II-induced pressor response in normotensive rats

In conscious rats, the pressor responses to intravenous administration of 100 ng/kg angiotensin II prior to drug administration were comparable among all groups [46±1 (n=5), 47±4 (n=4), 45±2 (n=4) and 45±2 (n=4) mm Hg following oral administration of 0.03, 0.1, 0.3 and 1 mg/kg azilsartan medoxomil, respectively, and 47±3 (n=5), 44±2 (n=4), 48±4 (n=4) and 46±2 (n=5) mm Hg following oral administration of 0.03, 0.1, 0.3 and 1 mg/kg olmesartan medoxomil, respectively]. In addition, baseline blood pressures were not significantly different among these groups [93±2 (n=5), 98±3 (n=4), 105±4 (n=4) and 99±6 (n=4) mm Hg following administration of 0.03, 0.1, 0.3 and 1 mg/kg azilsartan medoxomil, respectively, and 96±3 (n=5), 100±3 (n=4), 100±1 (n=4) and 104±1 (n=5) following administration of 0.03, 0.1, 0.3 and 1 mg/kg olmesartan medoxomil, respectively].

Oral administration of 0.03, 0.1, 0.3 and 1 mg/kg azilsartan medoxomil inhibited the angiotensin II-induced pressor response in a dose-related manner (Fig. 3A). The maximum response to each dose was attained 5–7 h after administration; the respective inhibition rates were 26±7 (n=5), 64±9 (n=4), 80±6 (n=4) and 100±0 (n=4)%. Azilsartan medoxomil-induced inhibitory effects were observed 24 h after administration of 0.1, 0.3 and 1 mg/kg, and the respective inhibition rates were 31±13 (n=4), 53±6 (n=4) and 91±4 (n=4)%.

Oral administration of 0.03, 0.1, 0.3 and 1 mg/kg olmesartan medoxomil also exerted inhibitory effects on the angiotensin II-induced pressor response in rats (Fig. 3B). The pressor response was inhibited by 13±5% (n=5) 24 h after administration of the highest olmesartan medoxomil dose tested (1 mg/kg). The ID_{50} value for azilsartan medoxomil was determined to be 0.12 mg/kg, which is approximately four times lower than that calculated for olmesartan medoxomil (0.55 mg/kg).

Direct comparisons of the effects of olmesartan medoxomil and azilsartan medoxomil at 1 mg/kg indicate that the in vivo antagonistic activity of azilsartan medoxomil is significantly superior to that of olmesartan medoxomil ($P \leq 0.05$, Student's *t*-test) in terms of the inhibition rate at 24 h after administration and the $AUC_{0-24\ h}$ value (2276±23 mm Hg×h for azilsartan medoxomil and 1411±91 mm Hg×h for olmesartan medoxomil).

3.3. Acute antihypertensive effects in SHRs

Baseline mean blood pressures were 168±4 (n=6), 166±2 (n=7), 170±2 (n=7), 170±4 (n=5), 167±4 (n=7), 167±3 (n=7), 169±5 (n=4) and 168±4 (n=5) mm Hg for the groups administered the vehicle, 0.1, 0.3 and 1 mg/kg azilsartan medoxomil or 0.1, 0.3, 1 and 3 mg/kg olmesartan medoxomil, respectively. Baseline heart rates were 275±3 (n=6), 276±6 (n=7), 271±7 (n=7), 308±16 (n=5), 286±11 (n=7), 279±11 (n=7), 273±17 (n=4) and 272±7 (n=5) bpm for the groups administered the vehicle, 0.1, 0.3 and 1 mg/kg azilsartan medoxomil or 0.1, 0.3, 1 and 3 mg/kg olmesartan medoxomil, respectively. No differences were observed among these groups in terms of the baseline mean blood pressure or heart rate.

Oral administration of 0.1, 0.3 and 1 mg/kg azilsartan medoxomil significantly reduced the mean blood pressure in a dose-related manner without inducing reflex tachycardia (Fig. 4). This antihypertensive effect developed gradually and became stable 7–10 h after drug administration. The reduction in the mean blood pressure lasted 24 h at every dose tested (Fig. 4A).

Oral administration of 0.1, 0.3, 1 and 3 mg/kg olmesartan medoxomil also produced dose-related reductions in the mean blood pressure without inducing tachycardia (Fig. 5). In contrast to the antihypertensive effects of azilsartan medoxomil, the effects of olmesartan medoxomil lasted 24 h only after administration of the two highest doses (Fig. 5A).

The ED_{25} value for azilsartan medoxomil was 0.41 mg/kg, which is approximately three times lower than that calculated for olmesartan medoxomil (1.3 mg/kg). Direct comparisons of the effects of the two compounds at 1 mg/kg indicate that the antihypertensive effects of azilsartan medoxomil are significantly superior to those of olmesartan medoxomil ($P \leq 0.05$, Student's *t*-test) in terms of blood pressure reduction at 24 h after administration (33±4 mm Hg for azilsartan medoxomil and 18±4 mm Hg for olmesartan medoxomil) and the $AUC_{0-24\ h}$ value (828±67 mm Hg×h for azilsartan medoxomil and 570±39 mm Hg×h for olmesartan medoxomil).

3.4. Acute antihypertensive effects in renal hypertensive dogs

Baseline systolic blood pressures were 208±1, 209±1, 211±1, 211±1 and 210±1 mm Hg for the groups (n=5 each) administered the vehicle, 0.1 and 1 mg/kg azilsartan medoxomil or 0.3 and 3 mg/kg olmesartan medoxomil, respectively. Baseline heart rates were 83±1, 87±2, 85±5, 84±5 and 93±4 bpm for the groups (n=5 each) administered the vehicle, 0.1 and 1 mg/kg azilsartan medoxomil or 0.3 and 3 mg/kg olmesartan medoxomil, respectively. No differences were observed among these groups in terms of the baseline systolic blood pressure or heart rate.

Oral administration of 0.1 and 1 mg/kg azilsartan medoxomil resulted in significant dose-related reductions in the systolic blood pressure (Fig. 6A). The maximum response to each dose was observed 3 h after administration, and the blood pressure-lowering effects induced by oral administration of 1 mg/kg azilsartan medoxomil lasted 24 h. The systolic blood pressure was reduced by 15±1 mm Hg at 24 h after administration (Fig. 6A). Azilsartan medoxomil did not induce reflex tachycardia at any of the doses tested (Fig. 6B).

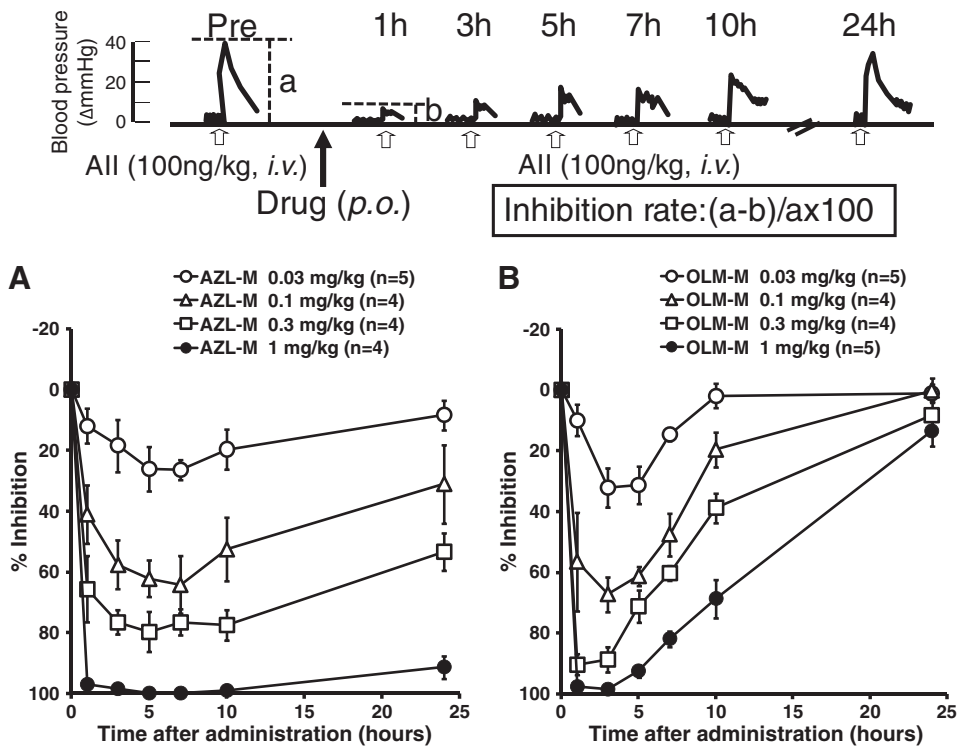

Fig. 3. Schematic diagram of the experimental protocol and inhibitory effects of azilsartan medoxomil (AZL-M) (A) and olmesartan medoxomil (OLM-M) (B) on the angiotensin II-induced pressor response in conscious rats. Angiotensin II (100 ng/kg) was intravenously injected before and 1, 3, 5, 7, 10 and 24 h after drug administration. The inhibition rate after treatment was calculated as described in the diagram. Values represent the mean ± S.E.M.

Oral administration of 0.3 and 3 mg/kg olmesartan medoxomil also led to dose-related reductions in the systolic blood pressure without inducing reflex tachycardia (Fig. 6). The maximum response to each dose was also observed 3 h after administration. Although the blood pressure-lowering effects induced by oral administration of 3 mg/kg olmesartan medoxomil lasted 24 h, the systolic blood pressure was reduced only by 4 ± 1 mm Hg from the baseline at 24 h after administration (Fig. 6A).

Comparison of the effects of the highest doses of each compound administered demonstrates that 1 mg/kg azilsartan medoxomil reduced the systolic blood pressure more potently than 3 mg/kg olmesartan medoxomil ($P \leq 0.05$, Student's *t*-test) in terms of blood pressure reduction at 24 h after administration and the $AUC_{0-24\ h}$ value (542 ± 22 mm Hg × h for azilsartan medoxomil and 350 ± 12 mm Hg × h for olmesartan medoxomil).

3.5. Long-term antihypertensive effects in conscious SHRs

Baseline systolic blood pressures were 214 ± 6 (n = 7), 214 ± 5 (n = 6), 214 ± 5 (n = 7), 213 ± 4 (n = 7), 212 ± 6 (n = 7), 214 ± 4 (n = 7) and 214 ± 3 (n = 7) mm Hg for the groups administered the vehicle, 0.1, 0.3 and 1 mg/kg azilsartan medoxomil or 1, 3 and 10 mg/kg olmesartan medoxomil, respectively. Baseline heart rates were 296 ± 19 (n = 7), 301 ± 14 (n = 6), 325 ± 9 (n = 7), 320 ± 12 (n = 7),311 ± 15 (n = 7), 317 ± 12 (n = 7) and 319 ± 7 (n = 7) bpm for the groups administered the vehicle, 0.1, 0.3 and 1 mg/kg azilsartan medoxomil or 1, 3 and 10 mg/kg olmesartan medoxomil, respectively. No notable differences were observed among these groups in terms of the baseline mean blood pressure or heart rate.

Repeated oral administration of azilsartan medoxomil once daily for 2 weeks significantly reduced the systolic blood pressure in a dose-related manner (Fig. 7A). The potency and duration of the antihypertensive effects of azilsartan medoxomil tended to increase with repeated doses compared with a single administration, and a stable reduction in the systolic blood pressure over a 24-h period was established 7 days after the start of repeated dosing. The minimum effective dose of azilsartan medoxomil was 0.1 mg/kg. Although the effects of azilsartan medoxomil on blood pressure gradually waned and the blood pressure returned to baseline after cessation of drug administration, significant blood pressure reductions were observed even 7 days after the final doses of 0.3 and 1 mg/kg were administered. No group experienced the rebound phenomenon (Fig. 7A). Azilsartan medoxomil did not have a significant effect on the heart rate (data not shown).

Olmesartan medoxomil also reduced the systolic blood pressure without inducing reflex tachycardia (Fig. 7B). The antihypertensive effects of olmesartan medoxomil were attenuated 24 h after administration compared with the effects observed at 5 h after administration. The antihypertensive effects of olmesartan medoxomil disappeared within 7 days after the final doses of 1 and 3 mg/kg were administered (Fig. 7B).

3.6. Effects on insulin sensitivity in SHRs

Pre-treatment values for body weight, plasma glucose, plasma insulin and systolic blood pressure were similar among all the groups studied. Four weeks of treatment with 0.3 and 1 mg/kg azilsartan medoxomil significantly increased the glucose infusion rate, an indicator of insulin sensitivity, in a dose-related manner (Fig. 8). Olmesartan medoxomil significantly improved the glucose infusion rate only when administered at a dose of 10 mg/kg (Fig. 8). Steady-state plasma insulin levels did not differ among the groups studied. These results suggest that azilsartan medoxomil improves insulin sensitivity in SHRs more potently than olmesartan medoxomil.

3.7. Antiproteinuric effects in Wistar fatty rats

Wistar fatty rats, a model of type 2 diabetes, developed proteinuria, hyperglycaemia and hyperinsulinaemia. At 25 weeks of age, prior to drug administration, the Wistar fatty rats were already

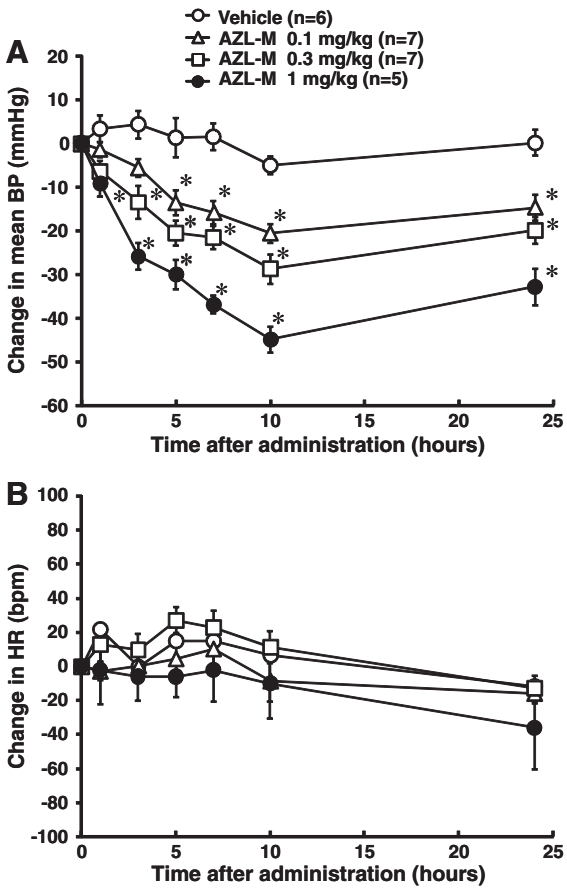

Fig. 4. Effects of oral administration of the vehicle or azilsartan medoxomil (AZL-M) on the mean blood pressure (A) and heart rate (B) in conscious SHRs. The blood pressure and heart rate were measured before and 1, 3, 5, 7, 10 and 24 h after oral administration. Values represent the mean ± S.E.M. *P ≤ 0.025 vs vehicle (one-tailed Williams test with Bonferroni correction).

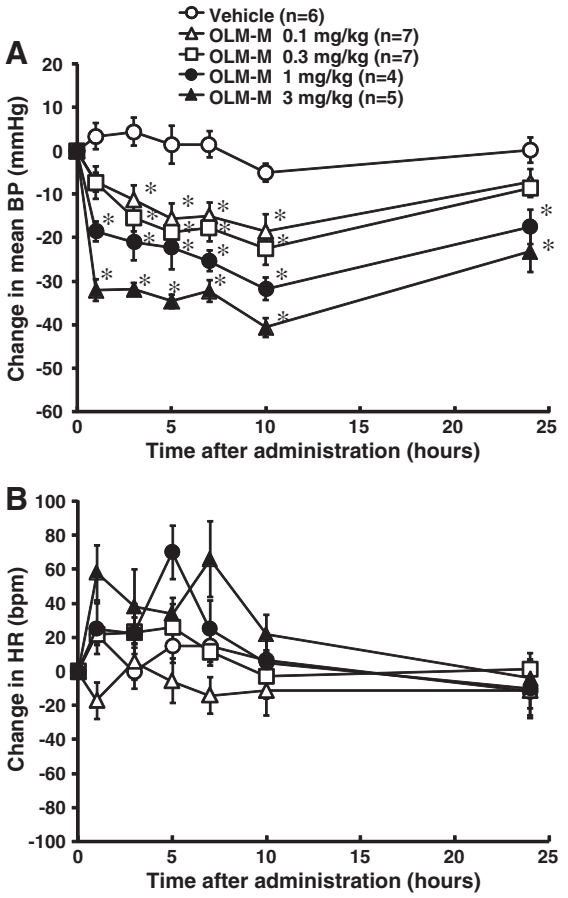

Fig. 5. Effects of oral administration of the vehicle or olmesartan medoxomil (OLM-M) on the mean blood pressure (A) and heart rate (B) in conscious SHRs. The blood pressure and heart rate were measured before and 1, 3, 5, 7, 10 and 24 h after oral administration. Values represent the mean ± S.E.M. *P ≤ 0.025 vs vehicle (one-tailed Williams test with Bonferroni correction).

showing markedly high urinary albumin and total protein excretion levels compared with Wistar lean rats (Fig. 9). In Wistar fatty rats, these excretion levels were further increased during the vehicle treatment. Although neither 0.3 mg/kg azilsartan medoxomil nor 3 mg/kg olmesartan medoxomil had an effect on proteinuria, both 1 mg/kg azilsartan medoxomil and 10 mg/kg olmesartan medoxomil significantly inhibited the progression of proteinuria in Wistar fatty rats (Fig. 9). Urinary albumin and total protein excretion levels tended to decrease after 4 weeks of treatment with 1 mg/kg azilsartan medoxomil and 10 mg/kg olmesartan medoxomil compared with pre-treatment levels. The antiproteinuric effects of 1 mg/kg azilsartan medoxomil and 10 mg/kg olmesartan medoxomil were similar. Thus, azilsartan medoxomil demonstrated more potent antiproteinuric effects than olmesartan medoxomil in Wistar fatty rats.

At the end of the experiment (29 weeks of age), body weight, water intake and food intake were higher for Wistar fatty rats than for Wistar lean rats [636 ± 6 g, 61 ± 9 ml/day, 77 ± 9 ml/day and 27 ± 2 g/day (n = 7) for Wistar fatty rats and 529 ± 14 g, 25 ± 4 ml/day, 44 ± 5 ml/day and 20 ± 2 g/day (n = 4) for Wistar lean rats, respectively (P ≤ 0.05, Student's *t*-test or Aspin–Welch test)]. Doses of 0.3 and 1 mg/kg azilsartan medoxomil had no significant effect on body weight, food or water intake or urinary volume. At 10 mg/kg, olmesartan medoxomil resulted in a slight but significant decrease in food intake and increase in body weight, whereas 3 and 10 mg/kg olmesartan medoxomil had no significant effects on water intake or urinary volume. At 29 weeks of age, plasma glucose, immunoreactive insulin, triglyceride and total cholesterol levels were higher in Wistar fatty rats than in Wistar lean rats [359 ± 17 mg/dl, 1366 ± 129 μU/ml, 341 ± 27 mg/dl and 209 ± 7 mg/dl (n = 7) for Wistar fatty rats and 124 ± 7 mg/dl, 135 ± 6 μU/ml, 42 ± 1 mg/dl and 120 ± 6 mg/dl (n = 4) for Wistar lean rats, respectively (P ≤ 0.05, Student's *t*-test or Aspin–Welch test)]. Doses of 0.3 and 1 mg/kg azilsartan medoxomil and 3 and 10 mg/kg olmesartan medoxomil had no effects on these levels.

4. Discussion

In the present study, azilsartan medoxomil induced more potent and longer-lasting antihypertensive effects than olmesartan medoxomil in conscious SHRs and renal hypertensive dogs. These antihypertensive effects were stable during repeated administrations. Furthermore, azilsartan medoxomil improved insulin sensitivity in SHRs and reduced urinary protein excretion more potently than olmesartan medoxomil. This study is the first report to characterise the advantageous properties of azilsartan medoxomil as a novel antihypertensive agent.

4.1. Potency and persistence of the antihypertensive effects of azilsartan medoxomil

Although angiotensin II receptor blockers are generally well tolerated, physicians sometimes consider that the extent to which they can reduce the blood pressure is insufficient. For example, Mori et al. (2006) reported that 40.3% and 34% of hypertensive patients achieved a target systolic/diastolic blood pressure of <140/90 mm Hg with monotherapy comprising an L-type calcium channel blocker or angiotensin II receptor blocker, respectively. This difference may be

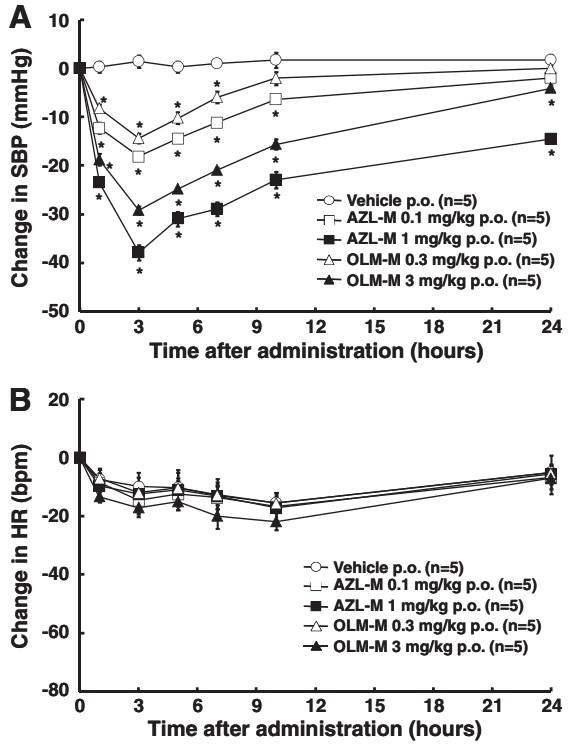

Fig. 6. Effects of oral administration of azilsartan medoxomil (AZL-M) and olmesartan medoxomil (OLM-M) on the systolic blood pressure (A) and heart rate (B) in conscious dogs with renal hypertension. The blood pressure and heart rate were measured before and 1, 3, 5, 7, 10 and 24 h after oral administration. Values represent the mean ± S.E.M. *P ≤ 0.025 vs vehicle (contrast test based on crossover ANOVA with Bonferroni correction).

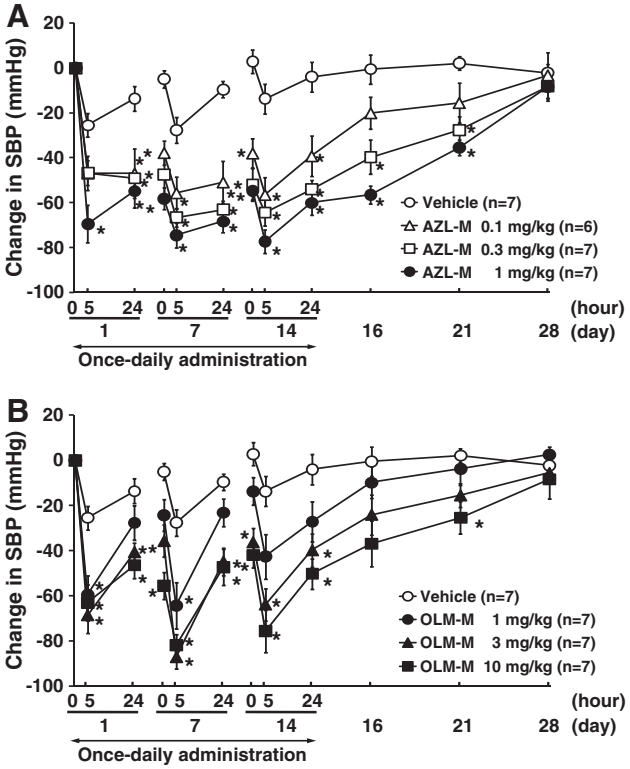

Fig. 7. Effects of repeated oral administration of azilsartan medoxomil (AZL-M) (A) and olmesartan medoxomil (OLM-M) (B) on the systolic blood pressure in SHRs. The blood pressure and heart rate were measured before and 5 and 24 h after drug administration on days 1, 7 and 14. To detect any potential rebounding, the systolic blood pressure and heart rate were also measured on days 2, 7 and 14 after the final dose was administered. Values represent the mean ± S.E.M. *P ≤ 0.025 vs vehicle (one-tailed Williams test with Bonferroni correction).

due to the limited contribution of endogenous angiotensin II to the maintenance of hypertension compared with L-type calcium channels. Alternatively, most angiotensin II receptor blockers may not completely inhibit the angiotensin AT_1 receptor at approved clinical doses. In the present study, azilsartan medoxomil induced more potent antihypertensive effects than olmesartan medoxomil in conscious SHRs (Figs. 4, 5 and 7) and renal hypertensive dogs (Fig. 6). These findings suggest that azilsartan medoxomil may prove to be a more complete antagonist against endogenous angiotensin II and may control blood pressure better than olmesartan medoxomil or other angiotensin II receptor blockers. Recent clinical studies on essential hypertensive patients have demonstrated that a significantly low blood pressure can be achieved with azilsartan medoxomil compared with olmesartan medoxomil or valsartan (White et al., 2011).

In addition to its antihypertensive potency, azilsartan medoxomil induced longer-lasting antihypertensive effects than olmesartan medoxomil in conscious SHRs and renal hypertensive dogs. Azilsartan medoxomil also induced a stable reduction in blood pressure over a 24-h period with repeated daily oral administrations (Fig. 7). This unique antihypertensive profile may be attributed to the long-lasting antagonistic activities of azilsartan medoxomil against endogenous angiotensin II in vivo, because azilsartan medoxomil demonstrated a stable inhibitory effect on the angiotensin II-induced pressor response throughout the day (Fig. 3). Investigators have reported that elevations in blood pressure around midnight and early morning are important predictors of central nervous system and cardiovascular outcomes in hypertensive patients (Kario, 2004; Mancia, 2005, 2007; Mancia et al., 2006). Given the results of the present study, azilsartan medoxomil is expected to be able to control the blood pressure for a 24-h period, which may contribute to the prevention of cardiovascular events.

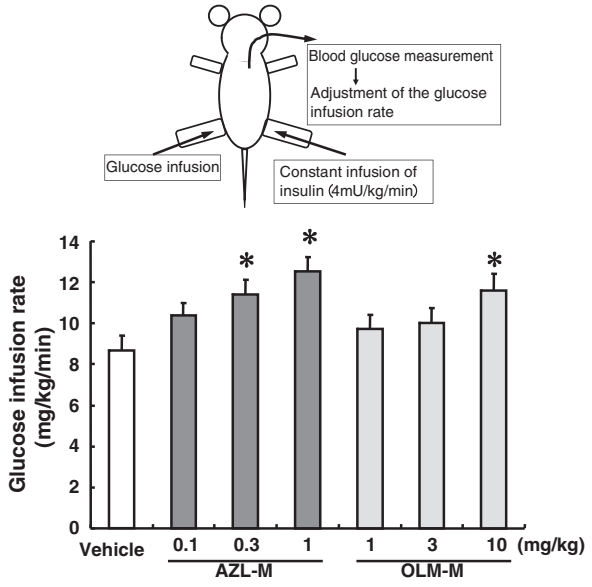

Fig. 8. Schematic diagram of the experimental protocol and effects of azilsartan medoxomil (AZL-M) and olmesartan medoxomil (OLM-M) on the glucose infusion rate, an indicator of insulin sensitivity, in SHRs. Glucose levels were measured from blood samples drawn from the right carotid artery and used for adjusting the glucose infusion rate. The glucose solution was infused into the left femoral vein and the insulin solution was infused into the right femoral vein throughout the experimental period. Values represent the mean ± S.E.M. *P ≤ 0.025 vs vehicle (one-tailed Williams test).

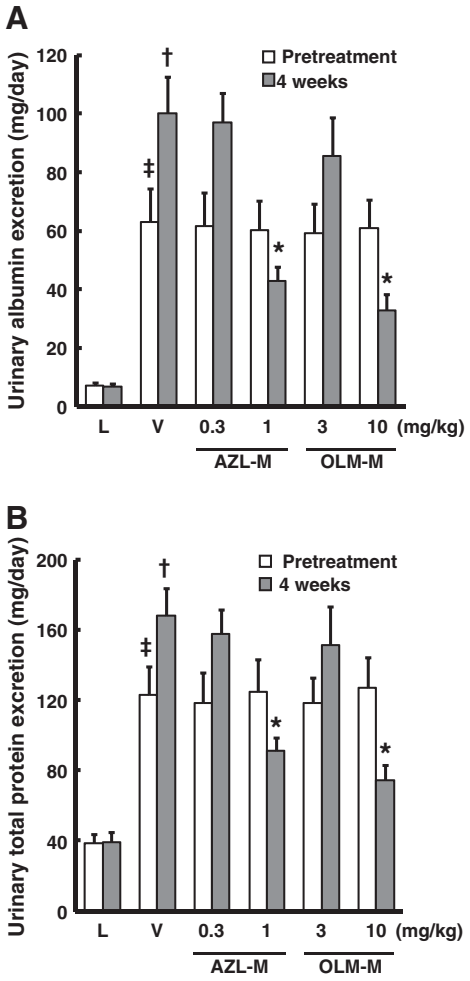

Fig. 9. Effects of azilsartan medoxomil (AZL-M) and olmesartan medoxomil (OLM-M) on urinary albumin (A) and total protein excretion levels (B) in Wistar fatty rats. Values represent the mean ± S.E.M. Drugs and the vehicle (V) were administered to Wistar fatty rats (n = 7). The vehicle was also administered to Wistar lean rats (L; n = 4). *P ≤ 0.025 vs vehicle (one-tailed Williams test). †P ≤ 0.01 vs Wistar lean rats. (Aspin–Welch test). ‡P ≤ 0.01 vs pre-treatment values in Wistar lean rats (Aspin–Welch test).

In the long-term study performed on SHRs, significant reductions in blood pressure lasted 7 days after administration of azilsartan medoxomil was stopped (Fig. 7A). The persistent durability of the antihypertensive effects of azilsartan medoxomil may minimise variations in blood pressure that could occur when doses are missed. This is often observed in clinical settings because hypertension does not generally induce symptoms until organ damage occurs (Fodor et al., 2005; Weir et al., 2000); therefore, this property of azilsartan medoxomil may also potentiate its effects on cardiovascular outcomes.

We previously measured plasma concentrations of azilsartan after oral administration of 1.33 mg/kg azilsartan medoxomil and observed that low plasma concentrations were achieved at 24 h after administration in normal rats and dogs; the plasma concentration of azilsartan reached a C_{max} of 1.0 μg/ml at 2 h after administration, but decreased to 0.094 μg/ml at 24 h after administration in rats. The plasma concentration reached a C_{max} of 0.62 μg/ml at 0.69 h after administration, but decreased to <0.009 μg/ml at 24 h after administration in dogs. In contrast, the inhibitory effect of azilsartan medoxomil against angiotensin II was maintained even 24 h after oral administration of 1 mg/kg azilsartan medoxomil (91% inhibition) in normal rats (Fig. 3A). In addition, a stable antihypertensive effect was observed at 24 h after administration in SHRs (Fig. 4A), and a significant reduction in blood pressure was observed at 24 h after administration of 1 mg/kg azilsartan medoxomil in renal hypertensive dogs (Fig. 6A). Thus, the pharmacokinetic properties of azilsartan medoxomil are not consistent with its in vivo antagonistic activities. Therefore, other mechanisms must contribute to the long-lasting effects of azilsartan medoxomil on blood pressure reduction.

One potential explanation for the prolonged antihypertensive effect of azilsartan medoxomil could be the binding profile of azilsartan to angiotensin AT_1 receptors, which is a proposed mechanism for angiotensin II receptor blockers that possess a carboxyl moiety at benzimidazole, such as CV-11974 (an active metabolite of candesartan cilexetil) (Noda et al., 1993; Ojima et al., 1997; Shibouta et al., 1993). CV-11974 has been reported to prevent angiotensin II-induced aortic vasoconstriction and maintain inhibitory activities even after washout. In contrast, losartan, which does not possess a carboxyl moiety, loses its inhibitory effect against angiotensin II after washout. Fierens et al. (2000) reported that Lys^{199} substitution in angiotensin AT_1 receptors results in the loss of its high affinity for CV-11974; therefore, the carboxyl group could interact with Lys^{199} to produce tight bonds at angiotensin AT_1 receptors. Azilsartan also possesses a carboxyl group that tightly binds to angiotensin AT_1 receptors, and its inhibitory effects against angiotensin II have been found to be persistent even after washout compared with those of other angiotensin II receptor blockers, including olmesartan, in experiments that used purified angiotensin AT_1 receptors, AT_1 receptor-expressing cells and isolated aortic vascular smooth muscles (Ojima et al., 2011). Therefore, the long-lasting antihypertensive effect of azilsartan medoxomil is most likely because of its tight anchoring to angiotensin AT_1 receptors.

Oral administration of azilsartan medoxomil did not induce reflex tachycardia associated with blood pressure reduction (Figs. 4, 6 and 7). Since similar results have been observed with other angiotensin II receptor blockers, angiotensin II may contribute to the induction of reflex tachycardia associated with blood pressure reduction by modifying the activation of cardiac sympathetic nerve systems and/or baroreceptor sensitivity (Inada et al., 1994). Although the heart rate was not measured in normal rats in this experiment, Inada et al. (1994) reported that other angiotensin II receptor blockers, such as losartan and candesartan cilexetil, do not induce reflex tachycardia but do induce small reductions in blood pressure in normal rats. Therefore, endogenous angiotensin II might not contribute to baroreflex sensitivity even under normal conditions. Alternatively, the slow onset of the antihypertensive effects of azilsartan medoxomil may attenuate the induction of reflex tachycardia, as reported using a sustained release formulation of a Ca^{2+} channel blocker and a slowly absorbed, ATP-sensitive K^+ channel opener (Kusumoto et al., 1994; Murdoch and Brogden, 1991).

4.2. Effects of azilsartan medoxomil on insulin resistance

Insulin resistance is associated with hypertension. Iimura et al. (1995) reported that candesartan cilexetil improves the insulin sensitivity of essential hypertensive patients, indicating the possible involvement of excess angiotensin II in the development of insulin resistance. SHRs also have an insulin resistance compared with normotensive rats, and candesartan cilexetil improves their insulin resistance (Iimura et al., 1995). The present study demonstrates that azilsartan medoxomil and olmesartan medoxomil produce dose-related improvements in the insulin sensitivity of SHRs (Fig. 8). These results indicate that endogenous angiotensin II contributes to the development of insulin resistance in SHRs, similar to hypertensive patients, and that SHR is a useful animal model for evaluating the effects of new antihypertensive agents. Since insulin resistance is a well-known risk factor for cardiovascular diseases (Haffner et al., 1990; Howard et al., 1996), the potent insulin-sensitising and blood pressure-lowering effects of azilsartan medoxomil are expected to be beneficial for treatment of hypertensive patients.

The mechanisms of angiotensin II that are involved in the pathogenesis of insulin resistance have been extensively studied in

recent decades. Stimulation of serine phosphorylation of insulin receptor substrate-1 (IRS-1) by angiotensin II is believed to be important for the inhibition of insulin-induced tyrosine phosphorylation of IRS-1, which is a critical step in the insulin signalling pathway. We have observed that azilsartan prevents angiotensin II-induced decreases in tyrosine phosphorylation of IRS-1 in primary cultures of rat skeletal muscle cells (unpublished observation). In addition, Iwai et al. (2007) demonstrated that azilsartan reduces the expression of tumour necrosis factor-α but increases the expression of adiponectin and peroxisome proliferator-activated receptor γ in type 2 diabetic mice. These mechanisms may contribute to the effects of azilsartan that were observed in SHRs. Further studies will be required to determine whether these mechanisms are reproducible in SHRs.

4.3. Effects of azilsartan medoxomil on proteinuria and/or albuminuria

Clinical and epidemiologic studies have reported that proteinuria and/or albuminuria are major risk factors for progression of end-stage renal and cardiovascular diseases (Agrawal et al., 2009). Furthermore, growing evidence suggests that reduction and normalisation of proteinuria and/or albuminuria by drug treatment is associated with a decreased risk for adverse renal outcomes (e.g. doubling of serum creatinine levels and development of end-stage renal disease) (Ruggenenti et al., 2001). Therefore, urinary protein and/or albumin excretion levels are potential surrogate markers of renal outcomes.

Angiotensin II receptor blockers have been demonstrated to lower the urinary albumin excretion rate in both clinical (Parving et al., 2001) and experimental (Mizuno et al., 2006; Noda et al., 2001) studies, and improved renal outcomes have been observed in diabetic patients in large clinical trials (Brenner et al., 2001; Lewis et al., 2001). In the present study, azilsartan medoxomil induced a more prominent antiproteinuric effect than olmesartan medoxomil when more potent antihypertensive effects were observed (Figs. 4, 5, 7 and 9). Because azilsartan medoxomil reduces the blood pressure to a greater extent than olmesartan medoxomil in hypertensive patients (White et al., 2011), superior antiproteinuric effects could be expected when azilsartan medoxomil is compared with olmesartan medoxomil in hypertensive patients with diabetic nephropathy.

Azilsartan medoxomil reduced urinary albumin and protein excretion levels without improving the metabolic parameters (Fig. 9). This finding suggests that its beneficial effects are directly exerted on the kidneys. The mechanisms involved in direct renoprotection by angiotensin II receptor blockers have been extensively investigated (Jefferson et al., 2008). Angiotensin II receptor blockers exert renoprotective effects by several mechanisms, including normalising glomerular capillary pressure, inhibiting podocyte injury, inhibiting proliferation of mesangial cells and inhibiting epithelial–mesenchymal transition of tubular cells. Although further analysis is needed, the inhibition of these mechanisms may be involved in renoprotective effects of azilsartan medoxomil and olmesartan medoxomil.

5. Conclusion

In animal models of hypertension and type 2 diabetes with nephropathy, azilsartan medoxomil was shown to be a long-lasting and orally active angiotensin AT_1 receptor blocker, and its antihypertensive, insulin-sensitising and antiproteinuric effects were superior to those of olmesartan medoxomil. These results suggest that azilsartan medoxomil could be a valuable angiotensin II receptor blocker for treatment of hypertensive patients.

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