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ORIGINAL ARTICLE

A small difference in the molecular structure of angiotensin II receptor blockers induces AT_1 receptor-dependent and -independent beneficial effects

Masahiro Fujino[1], Shin-ichiro Miura[1,2], Yoshihiro Kiya[1], Yukio Tominaga[3], Yoshino Matsuo[1], Sadashiva S Karnik[2] and Keijiro Saku[1]

Angiotensin II (Ang II) type 1 (AT_1) receptor blockers (ARBs) induce multiple pharmacological beneficial effects, but not all ARBs have the same effects and the molecular mechanisms underlying their actions are not certain. In this study, irbesartan and losartan were examined because of their different molecular structures (irbesartan has a cyclopentyl group whereas losartan has a chloride group). We analyzed the binding affinity and production of inositol phosphate (IP), monocyte chemoattractant protein-1 (MCP-1) and adiponectin. Compared with losartan, irbesartan showed a significantly higher binding affinity and slower dissociation rate from the AT_1 receptor and a significantly higher degree of inverse agonism and insurmountability toward IP production. These effects of irbesartan were not seen with the AT_1-Y113A mutant receptor. On the basis of the molecular modeling of the ARBs–AT_1 receptor complex and a mutagenesis study, the phenyl group at Tyr^{113} in the AT_1 receptor and the cyclopentyl group of irbesartan may form a hydrophobic interaction that is stronger than the losartan–AT_1 receptor interaction. Interestingly, irbesartan inhibited MCP-1 production more strongly than losartan. This effect was mediated by the inhibition of nuclear factor-kappa B activation that was independent of the AT_1 receptor in the human coronary endothelial cells. In addition, irbesartan, but not losartan, induced significant adiponectin production that was mediated by peroxisome proliferator-activated receptor-γ activation in 3T3-L1 adipocytes, and this effect was not mediated by the AT_1 receptor. In conclusion, irbesartan induced greater beneficial effects than losartan due to small differences between their molecular structures, and these differential effects were both dependent on and independent of the AT_1 receptor.
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INTRODUCTION

Angiotensin II (Ang II) type 1 (AT_1) receptor blockers (ARBs) are highly selective for the (AT_1 receptor, which is a member of the G protein-coupled receptor superfamily, and these agents block the diverse effects of Ang II. In addition to their blood pressure-lowering effects, ARBs provide cardiovascular and renal protection.[1] Many ARBs are available for clinical use, but recent clinical studies have shown that not all ARBs have the same effects;[2] therefore some of the benefits conferred by ARBs may not be class effects (common effect).[3–5] This notion of drug-specific effects is referred to as a 'molecular effect (off-target or drug effect)'. Most ARBs have a common chemical structure that includes a biphenyltetrazole group and an imidazole group. We previously reported that olmesartan has this common chemical structure, as well as a hydroxyl and a carboxyl group, and shows strong inverse agonism.[6] The interactions between the AT_1 receptor and the hydroxyl and carboxyl groups of olmesartan have an important role in inverse agonism. We hypothesized that small differences in the molecular structures among ARBs could lead to different degrees of inverse agonism. Small differences in the molecular structure of a ligand for a G protein-coupled receptor can lead to different pharmacological effects;[7,8] however, the molecular mechanisms of such receptor-dependent and -independent beneficial effects are not well understood.

Irbesartan inhibited basal production, as well as low-density lipoprotein- and platelet-activating factor-stimulated the monocyte chemoattractant protein-1 (MCP-1) production in isolated human monocytes, independent of Ang II stimulation.[9] In addition, irbesartan has been identified as a ligand of peroxisome proliferator-activated receptor (PPAR)-γ,[10] and irbesartan-induced adiponectin upregulation

[1]Department of Cardiology, Fukuoka University School of Medicine, Fukuoka, Japan; [2]Department of Molecular Cardiology, Lerner Research Institute, Cleveland Clinic Foundation, OH, USA and [3]Genomic Science Laboratories, Dainippon Sumitomo Pharma Company Limited, Osaka, Japan
Correspondence: Dr S Miura, Department of Cardiology, Fukuoka University School of Medicine, 7-45-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan.
E-mail: miuras@cis.fukuoka-u.ac.jp
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was observed in the absence of Ang II.[11] Thus, irbesartan may have beneficial effects independent of AT_1 receptor-mediated signaling. Because irbesartan was derived from losartan, both ARBs have common chemical structures (biphenyl-tetrazole and imidazole groups). However, irbesartan has a cyclopentyl group instead of the chloride group found in losartan. We speculated that this small difference between the molecular structures of these ARBs could induce both AT_1 receptor-dependent and -independent effects. To explore this hypothesis, we systematically examined the binding affinity to and dissociation from the AT_1 receptor, as well as the inverse agonism and insurmountability toward inositol phosphate (IP) production as AT_1 receptor dependent-effects and determined the unique binding behavior of irbesartan to the AT_1 receptor. In addition, we analyzed whether irbesartan inhibited MCP-1 production and adiponectin secretion from cells independent of the AT_1 receptor, and whether these effects were directly mediated by nuclear factor-kappa B (NF-κB) and PPAR-γ.

These experiments address the molecular mechanisms that may underlie the multiple pharmacologically beneficial effects induced by the small differences in the molecular structures of ARBs for the AT_1 receptor.

METHODS

Materials

The following antibodies and reagents were purchased: ARBs, irbesartan and losartan (Toronto Research Chemicals, Ontario, Canada); Ang II (Sigma-Aldrich, St Louis, MO, USA); ^{125}I-[Sar^1, Ile^8]Ang II (Amersham Biosciences, Buckinghamshire, UK); hygromycin and doxycycline (Clontech Laboratories, Mountain View, CA, USA) and geneticin (G418, MP Biomedics, Solon, OH, USA). The molecular structures of the ARBs are shown in Figure 1a.

Mutagenesis and expression of the AT_1 receptor and membrane preparation

The synthetic wild-type (WT) AT_1 receptor gene, cloned in the shuttle expression vector pMT-3, was used for expression and mutagenesis (Table 1), as described previously.[12]

Cell cultures, transfections and membrane preparation

COS1 cells, human coronary endothelial cells (HCECs) and mouse 3T3-L1 proadipocytes were cultured. COS1 cells were maintained in 10% fetal bovine serum and penicillin- and streptomycin-supplemented Dulbecco's modified Eagle's essential medium (Invitrogen, Carlsbad, CA, USA) in 5% CO_2 at 37 °C. The HCECs were grown in media. In these experiments, cells supplemented without cell-growth supplement were used. Cell viability was >95% by trypan blue exclusion analysisin control experiments. WT and mutant AT_1 receptors were transiently transfected into COS1 cells using Lipofectamine 2000 liposomal reagent (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instructions. Cell membranes were prepared by the nitrogen Parr bomb disruption method in the presence of protease inhibitors. In addition, mouse 3T3-L1 proadipocytes were cultured and differentiated as previously described[13] using a standard differentiation mixture (dexamethasone, 3-isobutyl-methylxanthine, insulin and 10% fetal bovine serum).

Tetracycline-inducible system using HEK293 cells expressing the WT AT_1 receptor

A tetracycline-inducible (Tet-ON) gene expression system was used in HEK293 cells stably transfected with the WT AT_1 receptor (Clontech Laboratories). Briefly, stably transformed HEK293 cells were transfected with the neomycin-resistant pTet-ON regulator plasmid encoding the reverse tetracycline-controlled transactivator (rtTA) protein. These stably transformed cells were grown in a medium containing 100 μg ml^{-1} G418. The Tet-ON inducible HEK293 cells were used for the transfection of WT AT_1 receptor-TRE-2-hyg plasmids with Lipofectamine 2000, and selected with 150 μg ml^{-1} hygromycin. The transfected cells with TRE-2-hyg and WT AT_1 receptor-TRE-2-hyg plasmids were maintained in aa medium with 100 μg ml^{-1} G418 and 100 μg ml^{-1} hygromycin. Dose- and time-dependent experiments on stably transfected Tet-ON cells showed a maximal induction of the WT AT_1 receptor at 400 μg ml^{-1} doxycycline after 4 days in culture. Experiments used a pooled population of cells with the WT AT_1 receptor induced by 0, 100 and 400 μg ml^{-1} doxycycline for 4 days.

Competition binding study

The bindinf affinity (K_d) and maximal binding capacity (B_{max}) values for receptor binding were determined by ^{125}I-[Sar^1, Ile^8]Ang II-binding experiments under equilibrium conditions as previously described.[12]

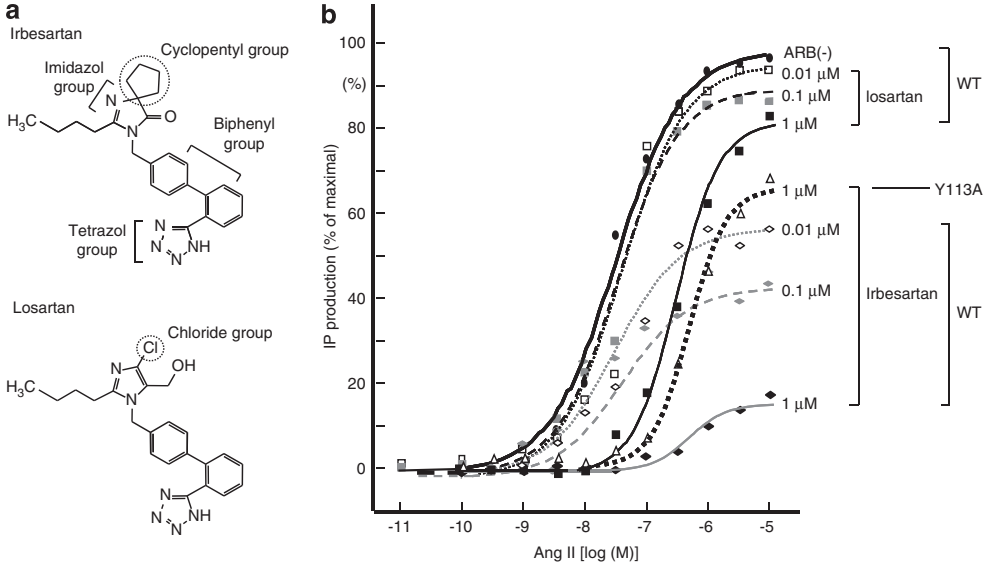

Figure 1 (**a**) Molecular structures of irbesartan and losartan. (**b**) The effect of preincubation with either irbesartan or with losartan on Ang II-mediated inositol phosphate (IP) production in COS cells with transiently transfected wild-type (WT) and Y113A AT_1 receptors. Cells were preincubated with or without the indicated concentrations of irbesartan or losartan for 30 min at 37 °C, and then further incubated for 5 min with increasing concentrations of Ang II. The percentage of maximal IP production in control cells without angiotensin II (Ang II) type 1 (AT_1) receptor blockers (ARBs) (ARB(−)) with WT and Y113A AT_1 receptors was adjusted to 100% (5934 ± 411 c.p.m. and 3502 ± 263 c.p.m., respectively).

Table 1 Maximal binding capacities (Bmax) and binding affinities (Kd) of [Sar^1,Ile^8]Ang II, irbesartan and losartan to AT_1 wild-type (WT) and mutants receptors

		K_d (nM)		
Receptor	*B_{max} (pmol mg^{-1} protein)*	*[Sar^1,Ile^8]Ang II*	*Irbesartan*	*Losartan*
WT	0.62 ± 0.07	0.8 ± 0.3 (1.0)	1.9 ± 1.1 (1.0)	11 ± 3 (1.0)
V108A	0.60 ± 0.11	0.7 ± 0.4 (0.9)	19 ± 5 (10)	416 ± 149 (38)
S109A	0.51 ± 0.03	0.9 ± 0.6 (1.1)	2.1 ± 0.6 (1.1)	33 ± 13 (3.0)
L112A	0.57 ± 0.04	0.8 ± 0.3 (1.0)	10 ± 1 (5.3)	593 ± 88 (54)
Y113F	0.71 ± 0.12	1.8 ± 1.0 (2.3)	4.6 ± 0.7 (2.4)	179 ± 18 (10)
Y113A	0.30 ± 0.02	0.8 ± 0.2 (1.0)	49 ± 5 (26)	1455 ± 431 (132)
Y184F	0.65 ± 0.04	0.9 ± 0.4 (1.1)	3.0 ± 1.3 (1.6)	40 ± 17 (3.6)
Y184A	0.56 ± 0.03	0.9 ± 0.3 (1.1)	2.1 ± 1.1 (1.1)	21 ± 3 (1.9)
K199Q	0.25 ± 0.04	2.0 ± 0.9 (2.5)	2.0 ± 1.0 (1.1)	21 ± 9 (1.9)
N200A	0.29 ± 0.02	1.8 ± 1.2 (2.3)	1.8 ± 0.2 (0.9)	26 ± 13 (2.4)
F204A	0.15 ± 0.02	24 ± 9 (30)	—	—
H256A	0.29 ± 0.09	0.8 ± 0.4 (1.0)	2.4 ± 1.4 (1.3)	41 ± 17 (3.7)
Q257A	0.15 ± 0.07	5.0 ± 2.1 (6.3)	3.2 ± 0.7 (1.7)	148 ± 29 (13)
M284G	0.43 ± 0.01	0.6 ± 0.1 (0.8)	0.7 ± 0.1 (0.4)	7.8 ± 2.3 (0.7)
M284A	0.41 ± 0.03	1.3 ± 0.7 (1.6)	0.5 ± 0.1 (0.3)	9.0 ± 0.9 (0.8)

IP production study
Total soluble IP was measured by the perchloric acid extraction method, which was described previously.[12]

Dissociation study by washing-out
Prepared cell membranes expressing the WT and mutant AT_1 receptors were incubated for 30 min at 22 °C with or without the indicated concentrations of ARBs. After the membranes were washed-out 1–3 times with excess cold phosphate-buffered saline, they were centrifuged for 10 min at 16 000 *g* at 4 °C. The membrane fractions were used in the assay for the specific binding of ^{125}I-[Sar^1, Ile^8]Ang II. The percentage of ARB dissociated from the AT_1 receptor was calculated by the following formula: 100−((specific binding using cell membrane without ARB treatment with no wash-out)−(specific binding using cell membrane with ARB treatment at the indicated wash-out times)/(specific binding using cell membrane without ARB treatment with no wash-out)−(specific binding using cell membrane with ARB treatment with no wash-out))×100 (%).

Molecular modeling of AT_1 receptor-ARBs
A binding model of irbesartan or losartan with the AT_1 receptor was constructed. InsightII software (Accelrys, San Diego, CA, USA) was used to construct a homology model of the human AT_1 receptor. The structure of bovine rhodopsin (Protein Data Bank code 1U19)[14] was used as a template for modeling the AT_1 receptor. The primary sequences of the AT_1 receptor and bovine rhodopsin were aligned in a manner consistent with a previous report.[15] Based on this alignment, the AT_1 receptor model was constructed and then subjected to a simulated annealing protocol by means of the Modeller program.[16] We selected important amino-acid residues of the AT_1 receptor to bind to irbesartan by site-directed mutagenesis studies. Although keeping the results of the mutagenesis study in mind, we manually docked irbesartan in the AT_1-receptor model, and the ligand-receptor model was then energy-minimized using an OPLS_2005 force field. The model was further refined according to the Induced Fit Docking Procedure based on Glide 4.5 and Prime 1.6, as implemented in the Schrödinger software package (Schrödinger, LLC, Portland, OR, USA). A binding model of losartan with the AT_1 receptor was also constructed by the Induced Fit Docking procedure, but in this case, the structure of the AT_1 receptor was obtained from the refined irbesartan-bound AT_1 receptor model.

Measurement of MCP-1 production and NF-κB activation
The HCECs were grown under serum-free conditions for 24 h with or without the indicated concentrations of ARBs. MCP-1 secretion in the medium from HCECs was measured by an ELISA kit (R&D Systems, Minneapolis, MN, USA). In addition, nuclear extracts from HCECs were prepared and NF-κB activation was measured by EZ-DetectTM Transcription Factor Kits for NF-κB p50 or p65 (Pierce, Rockford, IL, USA).

Receptor cofactor assay system for PPAR-γ
A receptor cofactor assay using the indicated concentrations of ARBs was carried out using EnBio receptor cofactor assay system for PPAR-γ (EnBioTec Laboratories, Tokyo, Japan).

PPAR-γ DNA-binding activity
PPAR-γ DNA-binding activities were examined with the PPAR-γ transcription factor assay kit (Cayman Chemical Company, Ann Arbor, MI, USA) using nuclear extracts from 3T3L1 adipocytes after 11 days of differentiation with and without the indicated concentrations of ARBs.

Statistical analysis
Results are expressed as the mean ± s.d. of three or more independent trials. Significant differences in measured values were evaluated with an analysis of variance using Fisher's *t*-test and paired or unpaired Student's *t*-test, as appropriate. Statistical significance was set at <0.05.

RESULTS
Binding of irbesartan and losartan to WT and mutant AT_1 receptors
The K_d of irbesartan was significantly lower than that of losartan for WT AT_1 receptors (Table 1). Next, we selected candidate residues (Val^{108}, Ser^{109}, Leu^{112}, Tyr^{113}, Tyr^{184}, Lys^{199}, Asn^{200}, Phe^{204}, His^{256}, Gln^{257} and Met^{284} in the AT_1 receptor) for specific binding sites of irbesartan and losartan, based on the molecular model of the AT_1 receptor complex described by previous reports.[6,17–19] To determine the specific amino acids that bind to these two ARBs, we examined the binding affinities of ARBs to AT_1 receptors mutated at the candidate amino acids mentioned above. The expression levels of the WT and mutated AT_1 receptors were within the same order of magnitude. The affinities of [Sar^1, Ile^8]Ang II were almost the same in some of the mutants and decreased in other mutants, but they were not less than 1/10 the affinity for the WT AT_1 receptor, except for F204A. F204A was not used in further analyses because the mutation itself affected the conformation of the AT_1 receptor. The affinity of irbesartan was reduced by more than 10-fold in V108A and Y113A

receptors and fivefold in L112A receptor compared with the WT AT_1 receptor. These results suggest that Val^{108}, Leu^{112} and Tyr^{113} in the AT_1 receptor are involved in binding to irbesartan. However, losartan may bind to Val^{108} Lue^{112}, Tyr^{113} and Gln^{257} because the affinity of losartan was reduced by more than 10-fold in V108A, L112A, Y113A and Q257A receptors compared with the WT AT_1 receptor. Irbesartan, which has a chemical structure similar to that of losartan and a cyclopentyl group, did not show a reduction in binding affinity to the Y113F (only a 2.4-fold reduction) mutant compared with the WT AT_1 receptor. Losartan, which has a chloride group instead of the cyclopentyl group found in irbesartan, showed a greater than 10-fold reduction in affinity for the Y113F mutant receptor. Although irbesartan showed a significant loss (26-fold reduction) in binding affinity for the Y113A receptor, losartan showed an even greater loss in binding affinity for the Y113A receptor (132-fold reduction). These results indicate that Tyr^{113} in the AT_1 receptor is a key residue mediating the differences in the binding behavior between irbesartan and losartan.

Insurmountabilily of irbesartan and losartan in WT and Y113A AT_1 receptors

The insurmountability of irbesartan and losartan in WT and Y113A AT_1 receptors were tested, and these results are shown in Figure 1b. Preincubation of cells expressing WT AT_1 receptor for 30 min with irbesartan (0.01, 0.1 and 1 μM) decreased the maximal response to subsequently added Ang II. The maximal response with 1 μM losartan was significantly higher than that with the lowest concentration of irbesartan tested (0.01 μM). In addition, a marked rightward shift of the Ang II concentration–response curve was observed with an increasing irbesartan concentration (0.01, 0.1 and 1 μM), whereas a rightward shift was observed with 1 μM losartan. Interestingly, the marked rightward shift and significant decrease in the maximal response with 1 μM irbesartan in the WT AT_1 receptor were not observed with 1 μM irbesartan in the Y113A AT_1 receptor. Thus, irbesartan had a higher degree of insurmountability for the AT_1 receptor than losartan. The strong insurmountability with irbesartan was not observed in the Y113A AT_1 receptor, indicating that Tyr^{113} is important for the strong irbesartan-induced insurmountability.

Inverse agonism of irbesartan and losartan in WT and mutant AT_1 receptors

The inverse agonist activities of irbesartan and losartan in the WT and mutant AT_1 receptor were tested, and the results are shown in Figure 2. We previously reported that the mutant AT_1 receptor (N111G) had high basal activity in the absence of Ang II and may have mimicked the pre-activated state of the WT AT_1 receptor.[20,21] Only irbesartan significantly suppressed the basal IP production in WT and N111G AT_1 receptors, in a dose-dependent manner. Interestingly, the inverse agonism observed with 1 μM irbesartan was lost with the Y113A and N111G/Y113A AT_1 receptors, thereby indicating that Tyr^{113} was also important for the inverse agonism of irbesartan.

Dissociation of irbesartan and losartan from WT and mutant AT_1 receptors

The degree of dissociation of irbesartan and losartan from the WT and mutant AT_1 receptors was tested, and the results are shown in Figure 3. Irbesartan (0.1–1 μM) showed a lower dissociation than losartan after the first wash-out, whereas a high concentration of losartan (1 μM) totally dissociated from the WT and mutant AT_1 receptors. After three washing-out procedures, 1 μM irbesartan totally dissociated from the WT AT_1 receptor. Interestingly, the low dissociation rate with 1 μM irbesartan was lost in the mutant AT_1 receptor (Y113A), thereby indicating that Tyr^{113} is important for the reduced dissociation rate of irbesartan.

Molecular model of the interaction between irbesartan or losartan and the AT_1 receptor

We found that the interaction between the Tyr^{113} residue in the AT_1 receptor and irbesartan may be important for multiple pharmacological effects of irbesartan, such as the high-binding affinity, the slow dissociation rate and the high degree of inverse agonism and insurmountability compared with losartan. To gain further insight into the interactions of irbesartan and losartan with the AT_1 receptor, a combined approach that included homology modeling and a docking study were carried out (Figure 4).

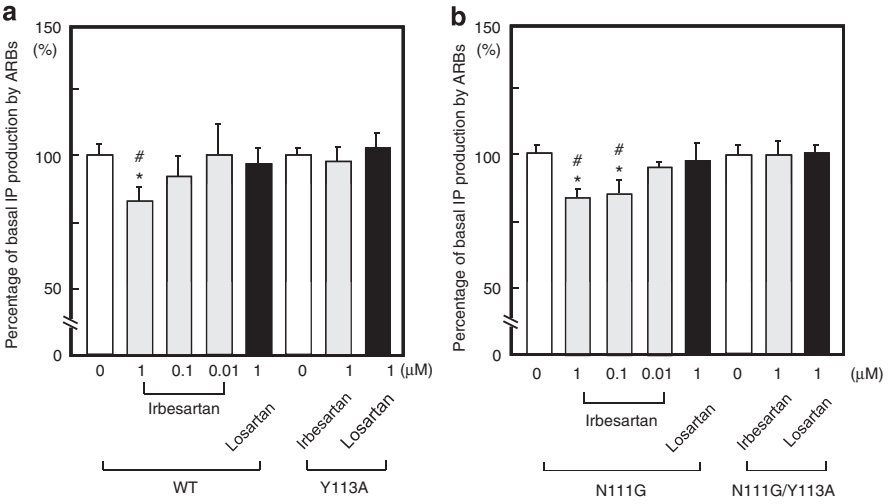

Figure 2 Percentage of the basal activity of inositol phosphate (IP) production with wild-type (WT) and Y113A AT_1 receptors (**a**) or N111G and N111G/Y113A AT_1 receptors (**b**) with the indicated concentrations of irbesartan and losartan. The percentage of basal activity without angiotensin II (Ang II) type 1 (AT_1) receptor blocker (ARB) treatment in WT (971 ± 84 c.p.m.), Y113A (947 ± 57 c.p.m.), N111G (2240 ± 98 c.p.m.) and N111G/Y113A (1650 ± 82 c.p.m.) AT_1 receptors were adjusted to 100%. Irbesartan and losartan were added 45 min before the measurement of IP. $^*P<0.05$ *vs.* no treatment in each AT_1 receptor. $^{\#}P<0.05$ *vs.* 1 μM losartan in each AT_1 receptor.

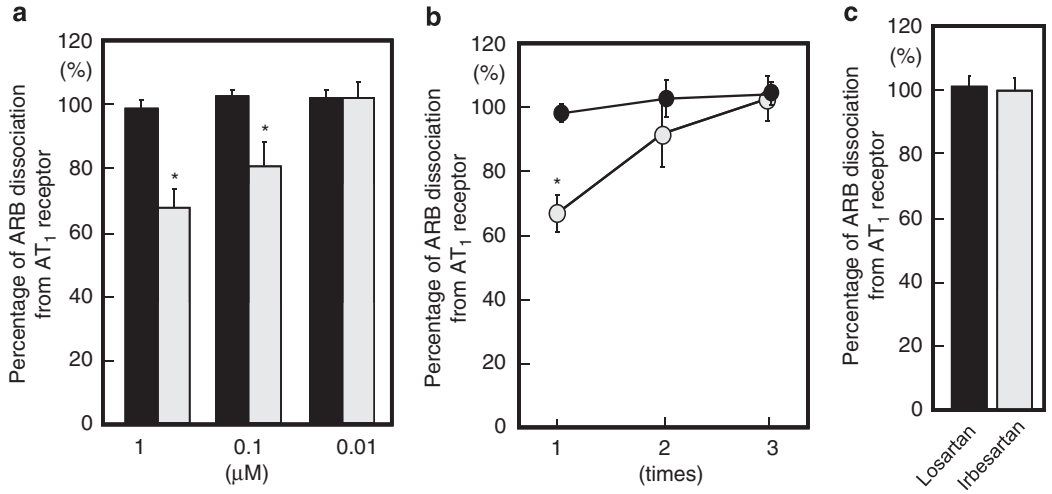

Figure 3 (**a**) Percentage of angiotensin II (Ang II) type 1 (AT_1) receptor blocker (ARB) dissociation from the WT AT_1 receptor after the first washing-out with the indicated concentrations of irbesartan and losartan. Closed and gray bars indicate losartan and irbesartan, respectively. *$P<0.05$ *vs.* losartan at the same concentration. (**b**) Percentage of ARB dissociation from the WT AT_1 receptor by 1–3 washing-out procedures with 1 μM irbesartan and losartan. Closed and gray circles indicate losartan and irbesartan, respectively. *$P<0.05$ *vs.* losartan at the same washing-out procedure. (**c**) Percentage of ARB dissociation from the Y113A AT_1 receptor by the first washing-out with 1 μM irbesartan and losartan. Closed and gray bars indicate losartan and irbesartan, respectively. NS, not significant.

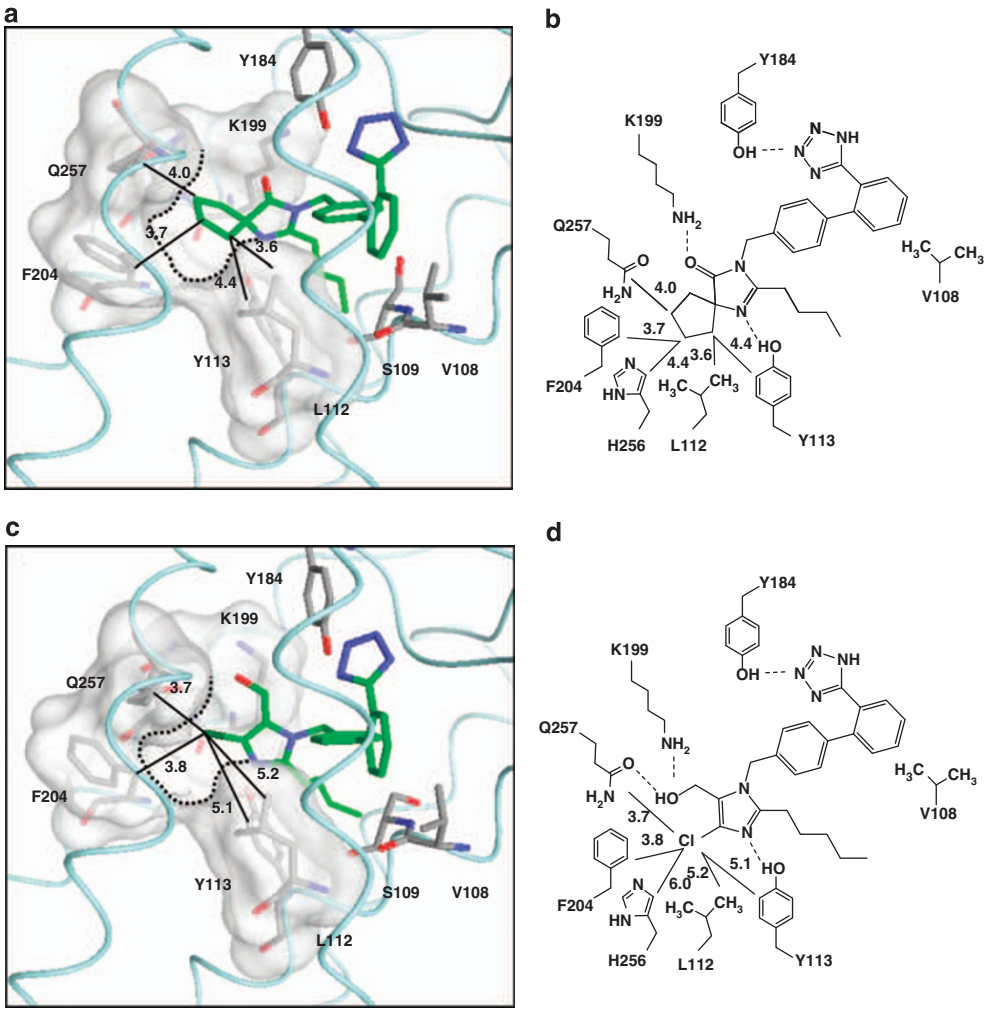

Figure 4 Molecular modeling of the interaction between irbesartan (**a**, **b**) or losartan (**c**, **d**) and the AT_1 receptor. The AT_1 receptor is shown as a loop and Val^{108}, Leu^{112}, Tyr^{113}, Tyr^{184}, Lys^{199}, Phe^{204}, Gln^{257} and irbesartan or losartan are shown as stick models. Close-up view of the interaction between irbesartan or losartan and the AT_1 receptor (**a**, **c**). Schematic drawing of the interaction between irbesartan or losartan and the AT_1 receptor (**b**, **d**). Dotted line indicates hydrophobic pocket of the AT_1 receptor.

According to site-directed mutagenesis studies, Val^{108}, Lue^{112} and Tyr^{113} in the AT_1 receptor have important roles in the binding of both irbesartan and losartan. In putative binding models, van der Waals (steric) interactions are observed between Val^{108} and the phenyl rings of both ARBs. The hydroxyl group of Tyr^{113} forms a hydrogen bond with the nitrogen at position three of the imidazolone ring of irbesartan and with the nitrogen at position three of the imidazole ring of losartan. In the Y113F mutant receptor, 2.4- and 10-fold decreases in K_d are seen for irbesartan and losartan, respectively. The decrease in the binding affinity of irbesartan for this mutant is rather small because Tyr^{113} interacts with irbesartan not only through hydrogen bonding but also by steric interactions. Tyr^{113} is located at the entrance of the hydrophobic pocket of the AT_1 receptor. This pocket is defined by Leu^{112}, Tyr^{113}, Phe^{204}, His^{256} and Gln^{257}, and accommodates the cyclopentyl group of irbesartan and the chlorine substituent of losartan. The shortest distances between the carbon atoms of the Leu^{112}, Tyr^{113}, Phe^{204}, His^{256} and Gln^{257} residues and the carbon atoms of the cyclopentyl group of irbesartan are 3.6, 4.4, 3.7, 4.4 and 4.0 Å, respectively. This indicates that the cyclopentyl group is tightly bound in the pocket. Although Tyr^{113} contributes to steric interactions with the cyclopentyl group of irbesartan, it may also help to maintain the shape of the pocket accommodating the cyclopentyl group because the side chains of Tyr^{113} and Leu^{112} are tightly packed. On the other hand, in the case of losartan, the shortest distances between the carbon atoms of the Leu^{112}, Tyr^{113}, Phe^{204}, His^{256} and Gln^{257} residues and the chloride atom of losartan are 6.2, 5.0, 6.4, 6.0 and 6.0 Å, respectively. This result suggests that the chloride atom is only loosely bound in the pocket.

Inhibition of MCP-1 production and NF-κB activation by irbesartan in HCECs and a Tet-ON system using HEK293 cells expressing the WT AT_1 receptor

Next, we analyzed whether irbesartan induced the inhibition of MCP-1 production independently of the AT_1 receptor in HCECs, and whether this effect was directly mediated by NF-κB (Figures 5a and b). Irbesartan inhibited MCP-1 production in a dose-dependent manner. The inhibition of MCP-1 production by 1 μM irbesartan was significantly higher than that with 1 μM losartan. In addition, 1 μM irbesartan significantly blocked NF-κB activation compared with 1 μM losartan.

The inhibition of both MCP-1 production and NF-κB activation in HCECs by irbesartan could be independent of the AT_1 receptor because AT_1 and AT_2 receptors were not found in HCECs according to competition binding studies (data not shown). To confirm this observation, we used a Tet-ON system using HEK293 cells expressing WT AT_1 receptor (Figures 5c and d). Because HEK293 cells do not endogenously express AT_1 and AT_2 receptors (data not shown), and we could analyze the activation using different expression levels of AT_1 receptor in the same cells, this system was a suitable surrogate model for linking the *de novo* expression of these receptors to MCP-1 production and NF-κB activation. The expression levels of AT_1 receptor after induction using 0, 100 and 400 μg ml^{-1} doxycycline

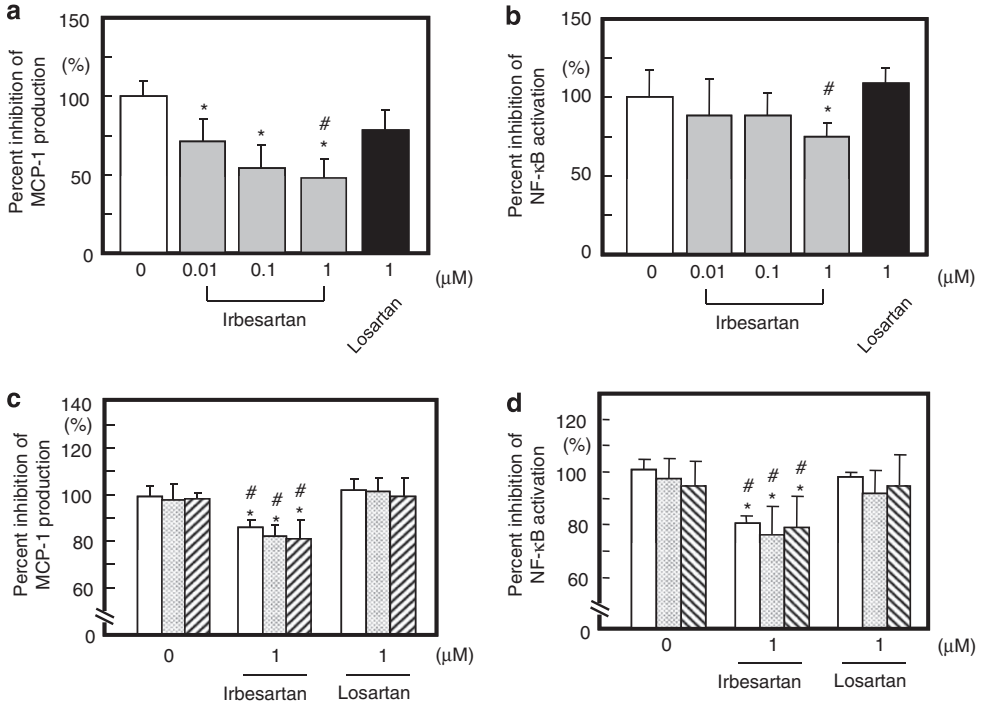

Figure 5 Percentage inhibition of monocyte chemoattractant protein-1 (MCP-1) production (**a**) and nuclear factor-kappa B (NF-κB) activation (**b**) by the indicated concentrations of irbesartan (gray bar) and losartan (closed bar) in human coronary endothelial cells (HCECs). HCECs were grown in the absence or presence of the indicated concentrations of angiotensin II (Ang II) type 1 (AT_1) receptor blockers (ARBs) under serum-free conditions for 24 h before the measurement of MCP-1 production and NF-κB activation. The percentage of basal MCP-1 production or NF-κB activation without ARB treatment under serum-free conditions for 24 h in HCECs was adjusted to 100%. $^*P<0.05$ *vs.* no treatment. $^\#P<0.05$ *vs.* 1 μM losartan. Percent inhibition of MCP-1 production (**c**) and NF-κB activation (**d**) by 1 μM irbesartan and losartan in a Tet-ON system using HEK293 cells expressing the WT AT_1 receptor. HEK293 cells were grown for 48 h using 0 (open bar), 100 (dotted bar) and 400 (stripe bar) mg ml^{-1} doxycycline for the induction of the WT AT_1 receptor. After induction, HEK293 cells were grown in the absence or presence of irbesartan and losartan under serum-free conditions for 24 h before the measurement of MCP-1 production and NF-κB activation. The percentage of basal MCP-1 production or NF-kB activation without ARB treatment under serum-free conditions for 24 h in HEK293 cells was adjusted to 100%. $^*P<0.05$ *vs.* no treatment. $^\#P<0.05$ *vs.* 1 μM losartan.

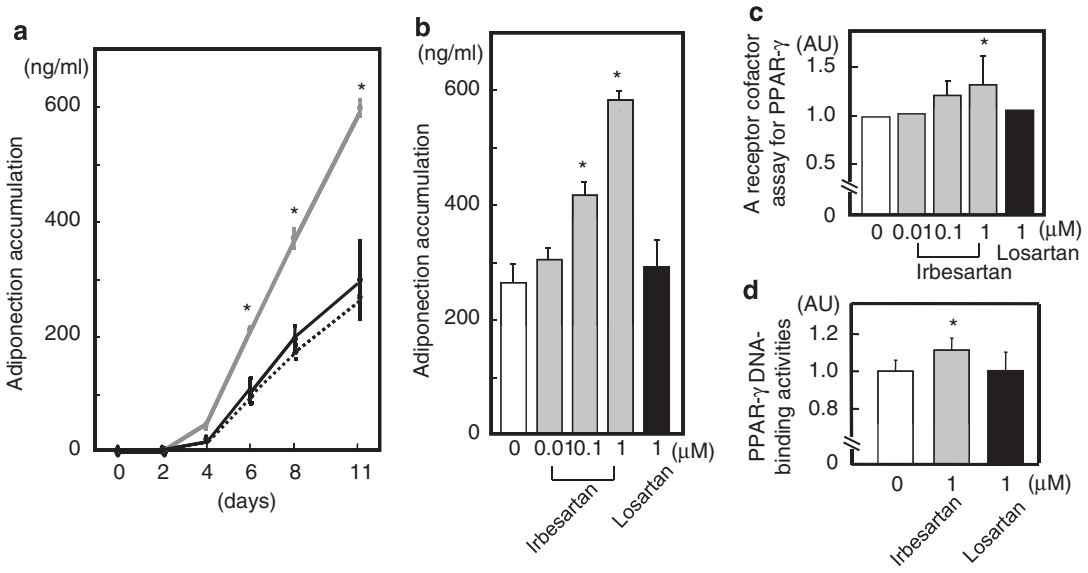

Figure 6 (**a**) Adiponectin accumulation in medium from 3T3L1 adipocytes after 2, 4, 6, 8 and 11 days of differentiation without (dotted line) and with 1 μM irbesartan (gray line) or 1 μM losartan (black line). *$P<0.05$ *vs.* no treatment and losartan at each time point. (**b**) Adiponectin accumulation in medium from 3T3L1 adipocytes after 11 days of differentiation without (open bar) and with 0.01, 0.1 and 1 μM irbesartan (gray bars) or 1 μM losartan (closed bar). *$P<0.05$ *vs.* no treatment and 1 μM losartan. (**c**) A receptor cofactor assay for peroxisome proliferator-activated receptor (PPAR)-γ using the indicated concentrations of angiotensin II (Ang II) type 1 (AT_1) receptor blockers (ARBs) (without (open bar) and with 0.01, 0.1 and 1 μM irbesartan (gray bars) or 1 μM losartan (closed bar)). *$P<0.05$ *vs.* no treatment and 1 μM losartan. (**d**) PPAR-γ DNA-binding activities in nuclear extracts from 3T3L1 adipocytes after 11 days of differentiation without (open bar) and with 1 μM irbesartan (gray bar) or 1 μM losartan (closed bar). *$P<0.05$ *vs.* no treatment.

were undetectable, 1.8 ± 0.1 and 4.5 ± 0.4 pmol mg^{-1} protein, respectively. Inhibition of MCP-1 production with 1 μM irbesartan was significantly higher than that with 1 μM losartan, which was independent of the expression levels of AT_1 receptor. In total, 1 μM irbesartan significantly inhibited MCP-1 production, independent of the expression levels of AT_1 receptor. In addition, 1 μM irbesartan, but not losartan, blocked NF-κB activation independently of the expression levels of AT_1 receptor.

Adiponectin secretion and PPAR-γ activation in 3T3-L1 adipocytes by irbesartan

Because irbesartan, but not eprosartan, was identified as a ligand of PPAR-γ and stimulated adiponectin protein expression,[22] we decided to compare irbesartan with losartan. As shown in Figures 6a and b, adiponectin was accumulated in 3T3-L1 cells after 11 days of treatment with irbesartan but not treatment with losartan. In addition, irbesartan stimulated adiponectin secretion in a dose-dependent manner. To evaluate the direct interaction between PPAR-γ and its co-factor in the presence of ARBs, as well as to distinguish whether an ARB was an agonist or antagonist, receptor cofactor assay system was performed (Figure 6c). The activity in 1 μM irbesartan was significantly higher than that with no treatment. In addition, PPAR-γ DNA-binding activity in nuclear extracts from 3T3L1 adipocytes with 1 μM irbesartan was significantly higher than from those with no treatment (Figure 6d). As a result, irbesartan induced significant PPAR-γ activation.

DISCUSSION

In this article, we provide direct evidence that small differences in the molecular structure of AT_1 receptor blockers (irbesartan and losartan) induced AT_1 receptor-dependent and -independent beneficial effects. Hypothetical irbesartan-induced AT_1 receptor-dependent and -independent beneficial effects are shown in Figure 7. Ang II binds to the AT_1 receptor and induces cell signaling, and subsequently stimulates cytokine and chemokine secretion, oxidative stress and cell proliferation, which eventually leads to cardiovascular disease. When irbesartan binds to the AT_1 receptor, its unique binding behavior to the AT_1 receptor led to a higher binding affinity, inverse agonism and insurmountability, and blocked AT_1 receptor-mediated signaling (AT_1 receptor-dependent). Irbesartan also has AT_1 receptor-independent beneficial effects (NF-κB/MCP-1 inhibition and PPAR-γ/adiponectin activation), and might bind to CCR2b and block MCP-1 binding.[23]

Many clinically important medications have been shown to behave as inverse agonists when tested against either WT or with mutated G protein-coupled receptors .[24,25] Spontaneous receptor mutations leading to constitutive activity have been implicated in some human diseases.[26,27] Although such spontaneous mutations have not been reported for the AT_1 receptor, we reported that the WT AT_1 receptor shows slight, but significant, constitutive activity.[28] A recent study showed that the WT AT_1 receptor is activated by the mechanical stretching of cultured rat myocytes[19,29,] and by constriction of the transverse aorta in angiotensinogen knock-out mice[29] without the involvement of Ang II; these adverse effects were suppressed by an inverse agonist. Thus, an inverse agonist for the AT_1 receptor may have pharmaco-therapeutical relevance for diseases of the cardiovascular system. We previously reported that the interactions between the hydroxyl group and carboxyl group of olmesartan and Tyr^{113} in the AT_1 receptor have important roles in the inverse agonist activity.[6] In addition, the most critical interaction for inducing inverse agonism of valsartan involved the interaction between the Lys^{199} of the AT_1 receptor and the tetrazole and phenyl groups of valsartan, even though its inverse agonism is comparable to that of olmesartan.[28] Although we indicated that the small differences in the molecular structure of ARBs could lead to differences in inverse agonism, the stronger hydrophobic interactions between irbesartan and the AT_1 receptor

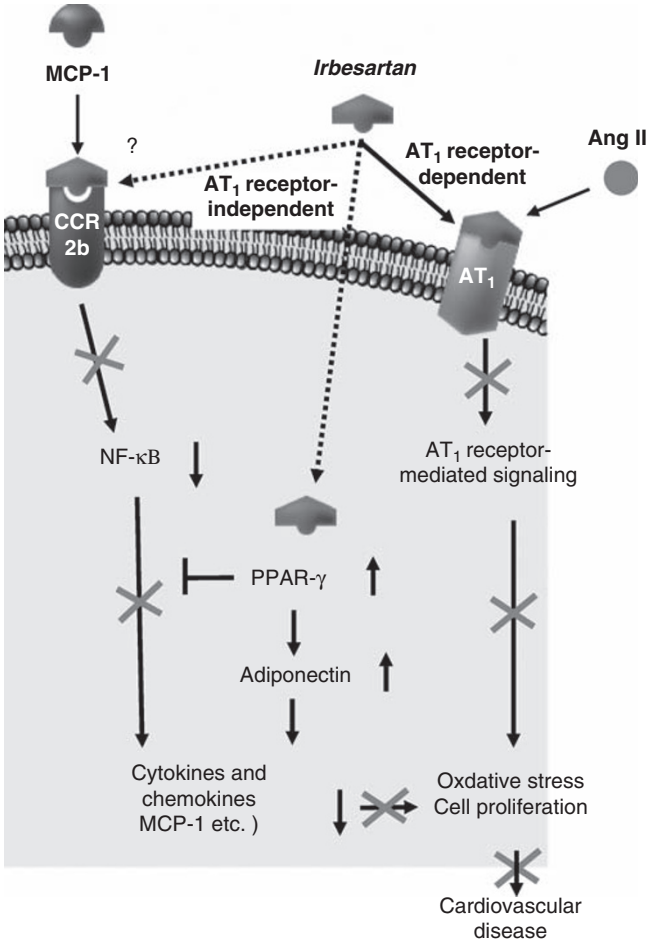

Figure 7 Hypothetical irbesartan-induced AT_1 receptor-dependent and -independent beneficial effects.

was important for inducing multiple pharmacological effects, such as a high-binding affinity and slow dissociation rate, as well as a high degree of insurmountability, and of inverse agonism. Thus, the effects of irbesartan were stronger than those of losartan. Because insurmountability was an Ang II-dependent effect and that inverse agonism was Ang II-independent, there was a difference in the pharmacological effects. Therefore, the specific hydrophobic interactions between irbesartan and the AT_1 receptor, mediated pharmacologically different effects, which involved Ang II-dependent and -independent pathways.

In this study, irbesartan inhibited MCP-1 production from HCECs independent of the AT_1 receptor and this effect may be mediated by NF-κB inactivation because HCECs do not express AT_1 or AT_2 receptors. The inhibition of basal MCP-1 production by irbesartan suggested two possible mechanisms. First, irbesartan may move into the cytoplasm and act directly on NFκB activity. We carried out a competition–binding study using a cytoplasmic fraction treated with irbesartan and losartan for 24 h, and found no specific ^{125}I-[Sar^1, Ile^8]Ang II binding in the cytoplasmic fraction (data not shown), thereby suggesting that the ARBs did not exist in the cytoplasm. Second, irbesartan may be able to bind to a receptor in the cell membrane other than the AT_1 receptor. Although a previous report indicated that irbesartan binds to platelet-activating factor receptor, the affinity of irbesartan for the platelet-activating factor receptor is 700 times less than that of platelet-activating factor.[9] Hence, some other membrane receptor may have a role in the irbesartan-induced inhibition of MCP-1 production. Interestingly, irbesartan and olmesartan may function as antagonists of the C–C Chemokine receptor, type-2b (CCR2b).[23] MCP-1 activated the pro-inflammatory transcription factors AP-1 and NF-κB, and enhanced the expression of its own mRNA in cells activated to express CCR2.[30] Because MCP-1 expression was dependent on NF-κB activation,[31] irbesartan could have blocked the binding of MCP-1 to CCR2b, and induced the inactivation of NFκB, which would have subsequently decreased the MCP-1 production in HCECs. In addition, Ang II could have activated NF-κB by AT_1 and AT_2 receptors.[32] Thus, if these cells expressed Ang II receptors, then ARBs could have blocked Ang II-induced NF-κB activation, and subsequently inhibited MCP-1 secretion.

Previous reports have indicated that irbesartan induced PPAR-γ activation and adiponectin secretion.[10,11] Although 3T3-L1 adipocytes expresses AT_1 and AT_2 receptors, Clasen *et al.*[11] reported that irbesartan-induced PPAR-γ activation was not AT_1 receptor-independent, but was AT_2 receptor-dependent.In addition, irbesartan and telmisartan influences the expression of PPAR-γ target genes in 3T3-L1 adipocytes.[33] According to the results of molecular-modeling experiments, the interactions of telmisartan with PPAR-γ may be explained by hydrophobic interactions. If so, telmisartan must directly activate PPAR-γ after passing through the cell membrane. In this study, we found that irbesartan moved into the cytoplasm based on our competition–binding study using a cytoplasmic fraction from 3T3-L1 adipocytes (data not shown). However, irbesartan did not move into the cytoplasm in HCECs, as we described above. Therefore, receptor cofactor assay system was carried out because it is a cell-free and a highly sensitive system. The results showed that irbesartan, but not losartan, was an agonist for PPAR-γ. Irbesartan induced PPAR-γ/adiponectin activation through an AT_1 receptor-independent pathway. Further studies are needed to confirm the mechanisms of irbesartan-induced activation independent of AT_1 receptor.

Most ARBs have common molecular structures (biphenyl-tetrazole and imidazole groups), and it is clear that ARBs have class effects. In addition, each ARB has been shown to have a molecular effect in basic experimental studies, including this and previous studies.[6,27,34] However, it is controversial whether each ARB would have a molecular effect in a clinical setting. For example, telmisartan, but not other ARBs, significantly induced PPAR-γ activation *in vitro*.[35] In clinical studies, changes in serum adiponectin and plasma glucose were significantly greater in a telmisartan group than in a candesartan group in patients with both type 2 diabetes and hypertension,[36] whereas candesartan therapy significantly lowered fasting insulin levels and increased plasma levels of adiponectin in patients with mild to moderate hypertension.[37] Although we understand that the molecular effects of each ARB in an experimental setting may not necessarily directly influence the clinical outcome, we believe that it is reasonable to consider the following possibility: a 100 mg dose of irbesartan results in human plasma irbesartan concentrations of approximately 1 μM,[38] and our results suggest that 1 μM of irbesartan induced beneficial effects in experimental studies.

Losartan is a prodrug, and *in vivo* cytochrome P450-mediated oxidation leads to formation of the metabolites Exp3174 and Exp3179. The molecular structures of Exp3174 and Exp3179 are slightly different than that of losartan. These metabolites also have unique beneficial effects. Although we do not know whether the small differences in the molecular structure between losartan and Exp3174 or Exp3179 are directly responsible for these effects, Exp3174 showed a higher capacity to bind the AT_1 receptor[21,] and Exp3179 abolished cyclooxygenase-2-mediated formation of thromboxane$_2$ and prostaglandin-$F_{2\alpha}$.[39] In this study, we compared the effects of losartan and

irbesartan because of their slight differences in molecular structures; however, we did not compare Exp3174 or Exp3179 with irbesartan. Further studies will be needed to clarify this point so that we do not exclude the beneficial effects of Exp3174 and Exp3179.

In summary, many clinical reports have discussed the varying degrees of beneficial effects of ARBs.[2] Some of the beneficial effects conferred by ARBs may be the molecular effects rather than the class effects. In this study, irbesartan induced more beneficial effects than losartan due to small differences in the molecular structures between these two ARBs, and these differences evoked AT_1 receptor-dependent and -independent beneficial effects. Although our findings regarding the molecular effects of ARB are based on basic research, these findings may lead to an exciting new area in clinical ARB treatment. A better understanding of the differential molecular mechanisms of each ARB could be helpful in the treatment of cardiovascular disease.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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