Distinct Properties of Telmisartan on Agonistic Activities for Peroxisome Proliferator-Activated Receptor γ among Clinically Used Angiotensin II Receptor Blockers: Drug-Target Interaction Analyses

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ABSTRACT

A proportion of angiotensin II type 1 receptor blockers (ARBs) improves glucose dyshomeostasis and insulin resistance in a clinical setting. Of these ARBs, telmisartan has the unique property of being a partial agonist for peroxisome proliferator-activated receptor γ (PPARγ). However, the detailed mechanism of how telmisartan acts on PPARγ and exerts its insulin-sensitizing effect is poorly understood. In this context, we investigated the agonistic activity of a variety of clinically available ARBs on PPARγ using isothermal titration calorimetry (ITC) and surface plasmon resonance (SPR) system. Based on physicochemical data, we then reevaluated the metabolically beneficial effects of telmisartan in cultured murine adipocytes. ITC and SPR assays demonstrated that telmisartan exhibited the highest affinity of the ARBs tested. Distribution coefficient and parallel artificial membrane permeability assays were used to assess lipophilicity and cell permeability, for which telmisartan exhibited the highest levels of both. We next examined the effect of each ARB on insulin-mediated glucose metabolism in 3T3-L1 preadipocytes. To investigate the impact on adipogenesis, 3T3-L1 preadipocytes were differentiated with each ARB in addition to standard inducers of differentiation for adipogenesis. Telmisartan dose-dependently facilitated adipogenesis and markedly augmented the mRNA expression of adipocyte fatty acid-binding protein (aP2), accompanied by an increase in the uptake of 2-deoxyglucose and protein expression of glucose transporter 4 (GLUT4). In contrast, other ARBs showed only marginal effects in these experiments. In accordance with its highest affinity of binding for PPARγ as well as the highest cell permeability, telmisartan superbly activates PPARγ among the ARBs tested, thereby providing a fresh avenue for treating hypertensive patients with metabolic derangement.

Introduction

Insulin resistance is frequently observed in hypertensive patients (Zavaroni et al., 1992; Lind et al., 1995; Lamouini-Zepter et al., 2006). Angiotensin II type 1 (AT1) receptor blockers (ARBs) are widely used for the treatment of hypertensive patients with fuel dyshomeostasis (Abuissa et al., 2005; Jandeleit-Dahm et al., 2005), as angiotensin II inhibits the physiologic differentiation of adipocytes and interferes with the insulin receptor signaling pathway via the AT1 receptor (Velloso et al., 1996; Janke et al., 2002). However, the degree of metabolically beneficial effects on insulin resistance or glucose dyshomeostasis differs among ARBs (Vitale et al., 2005; de Luis et al., 2010; Rizos et al., 2010).

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily of ligand-activated transcription factors. A variety of coactivators and corepressors are involved in the ligand-dependent transcription of genes, allowing tissue- and ligand-specific activation of target genes by PPARs. As a main subtype of PPARs, PPARγ controls a wide variety of genes involved in fuel storage in adipose

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ABBREVIATIONS: Ang II, angiotensin II; aP2, adipocyte fatty acid-binding protein; ARB, angiotensin II type 1 receptor blocker; AT1, angiotensin II type 1; DEX, dexamethasone; 2-DG, 2-deoxyglucose; DMEM, Dulbecco’s modified Eagle’s medium; DMSO, dimethylsulfoxide; FBS, fetal bovine serum; GLUT4, glucose transporter 4; IBMX, 3-isobutyl-1-methylxanthine; ITC, isothermal titration calorimetry; JIS, Japanese Industrial Standards; LBD, ligand-binding domain; PAMPA, parallel artificial membrane permeability; PBS, phosphate-buffered saline; PGC-1α, PPARγ-coactivator-1α; PPARγ, peroxisome proliferator-activated receptor γ; RT-PCR, real-time polymerase chain reaction; SPR, surface plasmon resonance; SRC-1, steroid receptor coactivator-1; T0070907, 2-chloro-5-nitro-N-4-pyridinyl-benzamide; TIF-2, transcriptional intermediary factor-2.
tissue and thus plays a crucial role in the regulation of adipogenesis and insulin sensitivity (Picard and Auwerx, 2002). It has been shown that relative ratio of mRNA level between PPARγ1 and PPARγ2 in 3T3-L1 preadipocytes is approximately 10-fold, but it turns out to be equal in differentiated 3T3-L1 adipocytes (Takenaka et al., 2013). It is also reported that PPARγ1 protein does express in 3T3-L1 adipocytes. PPARγ1 is expressed in preadipocytes, which increases approximately 8-fold during adipocyte differentiation. In contrast, PPARγ2 is slightly expressed in preadipocytes, which increases approximately 180-fold during adipocyte differentiation (Jitrapakdee et al., 2005). A wide variety of tissues or cells express PPARγ1, whereas PPARγ2 is highly restricted to adipocytes (Escher et al., 2001). PPARγ2 plays a crucial role in regulating the program of adipocyte differentiation (Tontonoz et al., 1994). Therefore, we evaluated the effect of telmisartan on PPARγ2 mRNA expression. A subgroup of ARBs has been reported to augment PPARγ activity (Benson et al., 2004; Schupp et al., 2004), suggesting that such ARBs could improve insulin resistance, at least in part, via this activity. However, despite attention being focused on the insulin-sensitizing effects of ARBs, the direct evidence of ARB interaction with PPARγ remains obscure.

Telmisartan, an ARB, has unique chemical properties that enable it to partially activate PPARγ (Amano et al., 2012) as well as strongly block AT1 receptors (Kakuta et al., 2005; Ohno et al., 2011). We recently demonstrated for the first time that telmisartan forms a ternary complex structure with PPARγ and a coactivator, steroid receptor coactivator-1 (SRC-1), thereby revealing that telmisartan directly binds to the ligand-binding domain of PPARγ via a mode distinct from that of full agonists for PPARγ such as rosiglitazone and farglitazar (Amano et al., 2012). In addition, telmisartan is known to induce a distinct subset of genes in adipocytes compared with PPARγ full agonists, indicating that it acts as a selective PPARγ modulator (SSPARM) (Schupp et al., 2005).

To clarify the mechanism by which telmisartan expresses agonistic activity for PPARγ, we focused on the direct interaction between ARBs and PPARγ. Two analytical methods— isothermal titration calorimetry (ITC) and surface plasmon resonance (SPR)—were used to precisely determine the binding affinities of ARBs for PPARγ. Further, to examine whether ARBs enable activation of PPARγ in cellular systems, we investigated physiochemical properties such as lipophilicity and cell permeability. Finally, by use of cultured murine adipocytes, 3T3-L1 cells, we assessed the impact of the interaction between ARBs and PPARγ on their PPARγ agonistic activities and the resultant insulin-sensitizing effects.

**Materials and Methods**

Clinically available angiotensin II AT1 receptor blockers were prepared as follows: telmisartan [2-(4-[4-methyl-6-(1-methyl-1H-1,3-benzodiazol-2-yl)-2-propyl-1H-1,3-benzodiazol-1-yl]methylene)benzoic acid] was purchased from Nippon Boehringer Ingenheim Pharmaceutics (Tokyo, Japan), and irbesartan [2-buty-3-(4-[2-(2HF-1,2,3,4-tetrazol-5-yl)phenyl]phenyl)methyl]-1,3-diazaspiro[4.4]nonane] was obtained from Shimogki Pharmaceutical (Tokyo, Japan). These compounds were then extracted by Astellas Pharmaceutical (Ibaraki, Japan). Azilsartan [2-ethoxy-1-(4-[2-(3,4-dihydroxy-1,2,4-oxadiazol-3-yl)phenyl]-4-yl)methyl]-1H-benzoimidazole-7-carboxylic acid] was synthesized by Astellas Pharmaceutical as previously reported elsewhere (Kohara et al., 1996). Candesartan [2-ethoxy-1-(4-[2-(2HF-1,2,3,4-tetrazol-5-yl)phenyl][phenyl)methyl]-1H-1,3-benzodiazole-7-carboxylic acid] was purchased from Sequoia Research Products (Pangrove, UK). A thiazolidinedione derivative, pioglitazone [(RS)-5.4-[2-(5-ethylpyridin-2-y)]ethoxylbenzylthiazolidine-2,4-dione], was purchased from Takeda Pharmaceutical (Osaka, Japan) and then extracted by Astellas Pharmaceutical. Farglitazar was synthesized by Astellas Pharmaceutical, as previously reported elsewhere (Henke et al., 1998). Human recombinant insulin was purchased from Funakoshi Co., Ltd. (Tokyo, Japan). We purchased 3-isobutyl-1-methoxyamine (IBMX), dexamethasone (DEX), and fetal bovine serum (FBS) from Sigma-Aldrich Japan (Tokyo, Japan). Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Life Technologies Japan (Tokyo, Japan). The lipid assay kit was purchased from Primary Cell (Hokkaido, Japan).

**Cell Culture and Oil Red O Staining.** 3T3-L1 cells were purchased from ATCC (Manassas, VA), maintained in DMEM supplemented with 10% FBS until they reached a state of density arrest at 2 days after confluence on 24-well plates, and then cultured and differentiated into adipocytes as described previously (Frost and Lane, 1985; Ishii-Yonemoto et al., 2010). In brief, cells were grown for 2 days after confluence (referred as day 0) in 10% FBS/DMEM. Differentiation was induced with 10% FBS/DMEM containing 0.5 mM IBMX, 0.25 μM DEX, and 1 μg/ml insulin for 2 days. The cells were then incubated in 10% FBS/DMEM with 1 μg/ml insulin for 2 days and maintained with 10% FBS/DMEM until day 6. Telmisartan, irbesartan, azilsartan, candesartan, and pioglitazone were dissolved in dimethylsulfoxide (DMSO) and added to media from days 0 to 6 with 0.1% of volume. Medium was changed every other day. On day 6, the cells were washed with phosphate-buffered saline (PBS) (−) twice, fixed in 3.7% formaldehyde for 1 hour at room temperature, and then stained with 0.6% (w/v) Oil Red O solution (60% isopropanol, 40% water) for 1 hour at room temperature. Cells were then washed with water to remove unbound dye. Oil Red O was eluted with isopropanol and quantified by measuring the optical absorbance at 510 nm (optical density 510) (Ramirez-Zacarias et al., 1992).

**PPARγ Protein Expression and Purification.** In comparison with PPARγ1, PPARγ2 contains 30 additional amino acids at its N terminus as a consequence of alternative splicing at the 5′-end of the gene (Tontonoz et al., 1994). It has been shown that PPARγ agonists bind to the both isoforms with a similar extent of IC50 and subsequently activate both isoforms in the same way (Elbrecht et al., 1996). It is noteworthy that the ligand binding domain of PPARγ has been subjected to a series of ITC experiments (Porcelli et al., 2012). DNA encoding human PPARγ ligand-binding domain (LBD) (amino acids 225–505) was amplified by PCR. For ITC studies, DNA was subcloned into the expression vector pET28a (Merek Millipore, Darmstadt, Germany). HisTag-PPARγ LBD was expressed in E. coli BL21(DE3) (Merek Millipore) and purified as previously described elsewhere (Amano et al., 2012). The target protein was dialyzed against buffer containing 20 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, 1 mM tris(2-carboxyethyl)phosphine hydrochloride, pH 8.0, and concentrated to 15 mg/ml. For SPR studies, DNA was subcloned into the modified expression vector pAN-4 (Cosmo Bio Co., Ltd, Tokyo, Japan), with an N-terminal HisTag followed by thrombin cleavage site and AviTag. HisTag-AviTag-PPARγ LBD was expressed in E. coli BL21(DE3) (Merek Millipore) transformed with pACYC184 harboring the birA gene. In vivo biotinylation of PPARγ LBD was performed through expression. The target protein was purified with a nickel-nitritotriacetic acid column, dialyzed against (20 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, and 1 mM Tris(2-carboxyethyl)phosphine hydrochloride, pH 8.0), and concentrated to 4 mg/ml.

**Isothermal Titration Calorimetry.** Isothermal titration calorimetry (ITC) experiments were performed using a high-performance MicroCal Auto-ITC200 system (GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, UK) (Velázquez-Campoy et al., 2004). All solutions contained within the calorimetric cell and injector syringes were prepared in the same buffer, which consisted of PBS, pH 7.4, with 5% DMSO. Binding affinities ($K_d = 1/K_i$) were determined
by injecting compounds (250 μM to 3 mM) into the calorimetric cell containing 2–25 μM PPARγ at 25°C. The absence of large particles (i.e., aggregates) in the compound solutions was confirmed using dynamic light-scattering equipment (Zetasizer Nano ZS, Malvern, UK) before the experiments (Yao et al., 2005). By using dynamic light-scattering, we detected possible compound aggregates in the pioglitazone solution, indicating the solubility of this compound in this experimental buffer was too low to determine the binding affinity with ITC. Therefore, we used farglitazar as a positive control. Each experiment was performed as one injection of 0.8 μl followed by 27 injections of 1.4 μl with a 200-second interval between each injection. The heat evolved after each injection was obtained from the integration of the calorimetric signal. Data were analyzed using a single binding site model implemented in the ORIGIN software package (OriginLab, Northampton, MA) provided with the instrument.

**Surface Plasmon Resonance.** SPR measurements were performed using a Biacore 4000 (GE Healthcare UK Ltd.) at 25°C. HBS-P+ buffer (10 mM HEPES, 150 mM NaCl, 0.05% Surfactant P20, pH 7.4) was used as the running buffer. Biotinylated PPARγ LBD was immobilized on the surface of a SA chip (GE Healthcare UK Ltd.) in the running buffer. ARBs at a range of concentrations were then injected into the flow cells at a flow rate of 30 μl/min for 60 seconds, followed by dissociation for 60 seconds. Data were analyzed using Biacore 4000 Evaluation Software version 1.0 for steady-state affinity analysis (Biacore, Uppsala, Sweden) to determine dissociation constants.

**Assays for Lipophilicity and Cell Permeability.** An octanol/pH 7.4 buffer distribution coefficient was used as a quantitative descriptor of lipophilicity of a series of ARBs. The procedure of distribution and separation was conducted by the Japanese Industrial Standards (JIS) protocol (JIS Z2600-107; OECD Test Guideline 107). The concentrations of the upper phase (octanol phase) and the lower phase (aqueous phase) were measured using a high-performance liquid chromatography/mass spectrometry system (Agilent Technologies, Palo Alto, CA).

A parallel artificial membrane permeability assay (PAMPA) was used to assess cell permeability in a series of ARBs (Avdeen, 2012). The PAMPA Evolution (pION Inc., Woburn, MA) using a double-sink PAMPA method was used in this study. In PAMPA, a “sandwich” is formed from a 96-well microtiter plate (PN 110243; pION Inc.) and a 96-well filter plate (IPVH; Millipore, Bedford, MA) such that each composite well is divided into two chambers: a donor at the bottom and an acceptor at the top. The donor solutions were adjusted to pH 6.5 (NaOH-treated universal buffer, PN 110151; pION Inc.), and the acceptor solutions were maintained at pH 7.4 (PN 110139; pION Inc.). After the formation of the sandwich, the plates were incubated at 25°C for 2 hours in a humidity-saturated atmosphere.

**Real-Time Polymerase Chain Reaction.** Total RNA was purified from cultured adipocytes on day 6 using an RNeasy Mini Kit (Qiagen, Tokyo, Japan) in accordance with the manufacturer’s instructions, and cDNA was synthesized as previously described elsewhere (Suwa et al., 2010). The level of mRNA expression of aP2 (adipocyte fatty acid-binding protein, which transports fatty acids into cells; the aP2 gene is a representative target of PPARγ) and PPARγ2 was quantified via real-time quantitative polymerase chain reaction (RT-PCR) using the SYBR Green method with a PRISM 7900 Sequence Detector (Applied Biosystems, Foster City, CA) provided with the instrument. The results were normalized to endogenous CATGAAGGA (reverse) and TGAGCCTCTGAAGTCCAGATA (forward) and ACAGGGTCTGGT- (Applied Biosystems, Foster City, CA). The chemiluminescence intensity was quantified using a quantitative digital imaging system (VersaDoc; Bio-Rad Laboratories). The amount was calculated as the percentage of activation (% of activation) compared with the DMSO control.

**Assay for 2-Deoxyglucose Uptake.** On day 6, glucose transport in 3T3-L1 adipocytes was assessed based on the uptake of 2-deoxy-[14C]glucose (PerkinElmer Japan, Kanagawa, Japan) as previously described elsewhere (Shimaya et al., 1998). In brief, cells were starved of serum for 5 hours before the experiments. Cultured adipocytes were washed with PBS, Krebs-Ringer-Hepes buffer (KRH buffer) or KRH buffer containing insulin (final concentration: 100 nM) then was added, and incubation was continued for 20 minutes. Glucose transport assays were initiated by the addition of 2-deoxy-[14C]glucose (final concentration: 200 μM, 0.2 μCi) for 10 minutes at 37°C. The reaction was terminated by washing with ice-cold PBS. Cells were disrupted with 0.5% SDS, and radioactivity was determined using a scintillation counter.

**Statistical Analysis.** Data are expressed as mean ± S.E.M. Results for lipid accumulation in cells, RT-PCR, glucose uptake, and GLUT4 protein expression were analyzed by analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test to compare the data between the vehicle (DMSO) and treated groups. P < 0.05 was considered statistically significant. All analyses were conducted using GraphPad Prism software (version 5.0, GraphPad, San Diego CA).

### Results

**Telmisartan Exhibited the Highest Affinity for PPARγ among Various Types of ARBs Tested.** The affinity ($K_d = 1/K_s$) of the interaction between PPARγ and ARBs was measured by ITC. In this experiment, the binding of farglitazar, a potent PPARγ full-agonist, was simultaneously assessed as a positive control. The binding affinities of a series of compounds are summarized in Table 1. Only telmisartan exhibited robust binding affinity for PPARγ, scoring a $K_d$ value in a nanomolar range ($K_d = 340 ± 40$ nM). In contrast, irbesartan showed marginal binding affinity for PPARγ ($K_d = 10.3 ± 1.5$ μM). The value of azilsartan and candesartan were both >100 μM.

The binding affinities for PPARγ as determined by SPR were as follows: telmisartan (8.6 ± 0.28 μM), irbesartan (60 ± 6.0 μM), azilsartan (>100 μM), and candesartan (>100 μM). The results obtained by SPR showed a markedly similar trend to those in ITC (Papalia et al., 2006). Both results are in agreement with previous reports of the rank order of PPARγ transactivation ability of ARBs (Benson et al., 2004; Schupp et al., 2004; Kajjya et al., 2011).

**Lipophilicities and Cell Permeability of ARBs.** Given that PPARγ is expressed in the nucleus, higher lipophilicity is required for ARBs to penetrate the lipophilic lipid bilayer (nuclear membrane, cell membrane) and interact with the PPARγ. Therefore, we assessed the lipophilicity and cell permeability of ARBs. Telmisartan exhibited the highest lipophilicity and the highest cell permeability of the compounds tested (Table 2). In contrast, irbesartan showed moderate lipophilicity and cell permeability. Azilsartan and candesartan showed subtle lipophilicity and cell permeability. Correlations between lipophilicity and cell permeability observed in this study are in good accordance with the previous report (Yamashita et al., 1997).
TABLE 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_d$ ($\mu$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Telmisartan</td>
<td>0.34 $\pm$ 0.04</td>
</tr>
<tr>
<td>Irbesartan</td>
<td>10.3 $\pm$ 1.5</td>
</tr>
<tr>
<td>Azilsartan</td>
<td>$&gt;100$</td>
</tr>
<tr>
<td>Candesartan</td>
<td>$&gt;100$</td>
</tr>
<tr>
<td>Farglitazar</td>
<td>0.01 $\pm$ 0.02</td>
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</table>

Effect of ARBs on GLUT4 Protein Level in 3T3-L1 Adipocytes. We assessed the effect of each ARB on GLUT4 protein expression in adipocytes. As shown in Fig. 4, telmisartan at 10 $\mu$M significantly increased GLUT4 protein by 3.0-fold. Likewise, irbesartan at 10 $\mu$M significantly increased GLUT4 protein expression by 2.8-fold. In contrast, azilsartan and candesartan did not increase GLUT4 protein expression.

TABLE 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>Lipophilicity</th>
<th>Cell Permeability ($\times 10^{-5}$ cm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Telmisartan</td>
<td>2.6 $\pm$ 0.0</td>
<td>27.1 $\pm$ 1.4</td>
</tr>
<tr>
<td>Irbesartan</td>
<td>1.3 $\pm$ 0.0</td>
<td>6.6 $\pm$ 0.5</td>
</tr>
<tr>
<td>Azilsartan</td>
<td>$-0.9$ $\pm$ 0.0</td>
<td>0.7 $\pm$ 0.2</td>
</tr>
<tr>
<td>Candesartan</td>
<td>$-1.5$ $\pm$ 0.0</td>
<td>0.4 $\pm$ 0.2</td>
</tr>
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</table>

Discussion

Affinity Analyses of ARBs to PPARγ. ITC and SPR are the latest techniques for characterizing the direct interaction of drugs and their target proteins (Renaud and Delsuc, 2009; Núñez et al., 2012; Holdgate et al., 2013). Both techniques can be used to determine the affinity ($K_d$) between a drug and its target protein in solution. ITC monitors thermal changes (i.e., thermodynamics) upon complex formation, and SPR monitors the process of association and dissociation between drugs and their target proteins. One advantage of ITC assays is that samples can be used without chemical modification, physical immobilization, or a combination of the two. Therefore, the interaction between a drug and its target protein can be elucidated in an environment that closely reflects physiologic conditions. To our knowledge, our present study is the first direct demonstration of the binding of each ARB for PPARγ.

By use of the ITC and SPR assays, we demonstrated that, among a series of ARBs, telmisartan exhibited the highest affinity for PPARγ. The binding affinities of ARBs show good correlation with transcriptional activities obtained from biochemical assays, indicating that the initial binding to PPARγ is a pivotal step in transcriptional activation.

The superior binding affinity of telmisartan for PPARγ may be attributable to its unique chemical structure. The molecular structure of telmisartan is divided into two parts that consist of biphenyl carboxylic acid (the A-part) and two benzimidazole rings (the B-part) (Fig. 5, inset). The A-part is conserved in most ARBs, but in telmisartan the carboxylic acid is substituted for bioisostere tetrazole. Further, the B-part of telmisartan is distinct from those of other ARBs (Fig. 5, inset).

We recently revealed the complex structure of PPARγ with telmisartan via X-ray crystallographic analysis (Amano et al., 2012). As shown in the Fig. 5 (inset), part of the two benzimidazole rings occupies a lipophilic narrow pocket surrounded by Ile281, Cys285, Leu356, Phe363, and His449 residues. In general, the binding of a compound to its target protein is mainly controlled by complementarity in shape and physicochemical properties (lipophilic and electrostatic characteristics). From this perspective, the benzimidazole rings of telmisartan might be...
advantageous for two reasons. First, the two benzimidazole rings, which can form a co-planner conformation, might fit into the narrow, flat pocket of PPAR\textsubscript{g}. Second, the lipophilic property of benzimidazole rings and the pocket surface of PPAR\textsubscript{g} are favorable for hydrophobic interaction, which is further reinforced by the stacking interaction between the benzene ring of the central benzimidazole ring and His449. It is noteworthy that the N3′ nitrogen of the central benzimidazole ring forms a hydrogen bond with Tyr473, which appears to be essential for PPAR\textsubscript{g} agonism (Amano et al., 2012). In contrast, other ARBs would be unable to bind PPAR\textsubscript{g} and form a hydrogen bond with Tyr473, in a similar fashion to telmisartan (Fig. 5, outset). For example, olmesartan and valsartan also lack benzene rings adjacent to the imidazole rings, which might reduce stacking interaction with His449. Taken together, the markedly high affinity of telmisartan for PPAR\textsubscript{g} observed via ITC and SPR can be attributed to the optimal complementarity in shape and lipophilicity.

**Physicochemical Properties of ARBs.** We have demonstrated for the first time that telmisartan exhibits the highest cell permeability of tested ARBs. Because PPAR\textsubscript{g} is localized in the nucleus, robust lipophilicity is critical for compounds to interact with the target through the highly lipophilic lipid bilayer (nuclear membrane and cell membrane). Consistent with a previous report (Yamashita et al., 1997), our study demonstrated good correlation between lipophilicity and cell permeability in each ARB. Indeed, when telmisartan was incubated with primary cultures of murine mesangial cells,
considerably high intracellular levels of the compound were detected by high-performance liquid chromatography analysis (Shao et al., 2007). In contrast, intracellular concentrations of losartan were undetectable in the same assay (Shao et al., 2007). This result also supports our finding that the concentration of telmisartan required to induce adipocyte differentiation is the lowest of the ARBs tested. In addition to the direct PPARγ agonistic properties, these data further suggest that the degree of lipophilicity of ARBs may also affect activating potency of PPARγ.

Fig. 2. Effect of ARBs and pioglitazone on the mRNA expression of aP2 and PPARγ2 in differentiating 3T3-L1 adipocytes. Cells were treated with each compound during the course of adipocyte differentiation (days 0–6). Total RNA was extracted, and mRNA for aP2 (A) or PPARγ2 (B) was determined via quantitative RT-PCR. Quantification of relative mRNA expression is shown. Results were normalized to the signal generated from β-actin mRNA. Data are expressed as mean ± S.E.M. from triplicate or quadruplicate experiments. **P < 0.01, ***P < 0.001 (Dunnett’s multiple comparison test) compared with vehicle (DMSO)-treated group. AZL, azilsartan; CAN, candesartan; IRB, irbesartan; PIO, pioglitazone; TEL, telmisartan.

Fig. 3. Effect of each ARB or pioglitazone on basal and insulin-stimulated 2-DG uptake in 3T3-L1 adipocytes. Cells were treated with each compound during the course of adipocyte differentiation (days 0–6). 2-DG uptake was measured in the absence (A) or presence (B) of 100 nM insulin. Data are expressed as mean ± S.E.M. from quadruplicate experiments. *P < 0.05, **P < 0.01, ***P < 0.001 (Dunnett’s multiple comparison test) compared with vehicle (DMSO)-treated group. AZL, azilsartan; CAN, candesartan; IRB, irbesartan; PIO, pioglitazone; TEL, telmisartan.
In addition to lipophilicity, the mode of action of telmisartan is pivotal in the activation of PPARγ. As reported by Schupp et al. (2004), telmisartan acts as a partial agonist for PPARγ. Crystallographic analysis revealed that telmisartan exhibits a distinct binding mode from full agonists for PPARγ (Amano et al., 2012). Binding of telmisartan to PPARγ results in unstable interaction with its helix 12, which may recruit a unique subset of coactivators. This binding pattern may also explain the mechanism by which telmisartan expresses a unique subset of genes. PPARγ-coactivator-1α (PGC-1α) binds to PPARγ to induce the expression of key enzymes involved in the mitochondrial respiration chain, thereby stimulating energy expenditure. SRC-1 stabilizes the binding of PGC-1α to PPARγ.

Notably, transcriptional intermediary factor-2 (TIF-2) competes with SRC-1 to form PGC-1α/PPARγ complexes, leading to an increase in PPARγ-mediated triglyceride storage within adipocytes (Picard et al., 2002). Therefore, an increase in the ratio of TIF-2 to SRC-1 in adipocytes might contribute to the progression of obesity and insulin resistance (Picard et al., 2002). A previous study revealed that telmisartan enhanced the transcriptional activation of PPARγ without recruitment of TIF-2. Telmisartan does activate adipogenesis through partial agonistic activity for PPARγ.

However, it should be noted that telmisartan-controlled balance between TIF-2 versus SRC-1 in adipocytes may be beneficial for the prevention of body fat accumulation (Schupp et al., 2005; Araki et al., 2006; Sugimoto et al., 2006; Shimabukuro et al., 2007). In contrast, PPARγ full agonists such as pioglitazone are associated with unwelcome weight gain via the robust recruitment of TIF-2. Weight reduction lowers arterial blood pressure in obese hypertensive patients, suggesting a close association between energy homeostasis and blood pressure (Busetto, 2001). A line of evidence has suggested that hypertrophic adipocyte-derived hypertensive substances, such as leptin and angiotensinogen, are involved in pathogenesis of obesity-related hypertension (Wajchenberg, 2000). Taken together, in telmisartan, less potency of body fat accumulation through partial agonistic activity for PPARγ as well as AT1-receptor blockade coordinate to improve hypertension in animals with elevated blood pressure.

It is noteworthy that the concentration of telmisartan required for the augmentation of lipid accumulation or glucose uptake in cultured adipocytes is achieved in the plasma of hypertensive patients treated with telmisartan but not with irbesartan (Marino et al., 1999; Israili, 2000). One exception was azilsartan, albeit only at high concentrations, which slightly but significantly augmented the lipid accumulation and mRNA level of PPARγ2 in a dose-independent manner. On

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**Fig. 4.** Effect of each ARB or pioglitazone on GLUT4 protein expression in 3T3-L1 adipocytes. Cells were treated with each compound during the course of adipocyte differentiation (day 0–6). On day 6, total cell lysates were prepared, and equal amounts of protein were subjected to Western blotting. Quantification of the relative protein expression was plotted. Data are expressed as mean ± S.E.M. from triplicate experiments. **P < 0.01 (Dunnett’s multiple comparison test) compared with vehicle (DMSO)-treated group. AZL, azilsartan; CAN, candesartan; IRB, irbesartan; PIO, pioglitazone; TEL, telmisartan."
the basis of these results, ARBs other than telmisartan might not exert PPARγ-dependent metabolically beneficial effects in hypertensive patients who were treated with conventional clinical dosage.

**Pharmacologic Properties of Telmisartan in Adipocytes and Its Clinical Implications.** With its prominent agonistic activity for PPARγ, telmisartan has been reported to improve insulin resistance and glucose dyshomeostasis both in humans and rodents (Benson et al., 2004; Rizos et al., 2009; Mori et al., 2011; Fujisaka et al., 2011; Takagi et al., 2013). Telmisartan has been reported to significantly decrease the plasma levels of fasting insulin and triglycerides, also to significantly decrease the value of HOMA-R (homeostasis model assessment for insulin resistance: index of insulin resistance), whereas telmisartan has been reported to significantly increase the plasma levels of adiponectin and high-density-lipoprotein cholesterol in diabetic patients with hypertension (Miura et al., 2005; Watanabe et al., 2010). Furthermore, systematic reviews of randomized clinical trials clearly demonstrate that telmisartan is superior to other ARBs in the amelioration of fasting plasma levels of glucose, insulin, triglyceride, and insulin resistance, which is accompanied by a significant increase in plasma adiponectin levels (Suksomboon et al., 2012; Takagi and Umemoto, 2012a,b,c).

A previous report demonstrated that telmisartan significantly increased serum adiponectin level in hypertensive patients with metabolic risk factors (Yano et al., 2007). Because adiponectin gene expression in adipocytes is strongly controlled by PPARγ (Maeda et al., 2001), it is reasonable to speculate that the partial agonistic activity for PPARγ in telmisartan at clinical doses significantly elevates the circulating level of adiponectin in humans. Adiponectin is shown to stimulate glucose use and fatty-acid oxidation by the activation of AMP-kinase, and subsequently to improve insulin resistance (Yamauchi et al., 2001, 2002). In this context, telmisartan would be beneficial for hypertensive patients who have multiple metabolic risk factors.

In our present study, telmisartan augmented 2-DG uptake in adipocytes at a concentration of 0.3 μM, comparable to that in the plasma of patients treated with the conventional clinical dosage. Furthermore, Furukawa et al. (2011) have reported that telmisartan but not candesartan increases the phosphorylation of insulin receptors, insulin receptor substrate-1, and protein kinase B by insulin in a “differentiated” 3T3-L1 adipocyte system, suggesting that telmisartan increases insulin sensitivity. In addition, those investigators also reported that up-regulation of glucose uptake by telmisartan was inhibited by the PPARγ antagonist T0070907 (2-chloro-5-nitro-N-4-pyridinylbenzamide), indicating that telmisartan acts via PPARγ activation in adipose tissue. We previously reported that telmisartan augmented the mRNA level of aP2 (a representative molecular indicator of PPARγ activation) in 3T3-L1 cells (Fujimoto et al., 2013).
used ARBs, telmisartan might solely exert PPARγ-dependent metabolically beneficial effects in clinical settings. As we describe in the Introduction, angiotensin II inhibits physiologic differentiation of adipocytes and interferes with insulin-receptor signaling via the AT1 receptor. Thus, in general, ARBs are considered to improve insulin resistance by the blockade of the AT1 receptor. Furthermore, telmisartan may also improve insulin resistance via PPARγ agonistic activity. Therefore, it is reasonable to speculate that telmisartan could outweigh the action of other ARBs (Fig. 6). Our findings provide novel insight into the therapeutic options for hypertensive patients who have insulin resistance and a convergence of metabolic risk factors.

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