Distinct Properties of Telmisartan on Agonistic Activities for Peroxisome Proliferator-Activated Receptor $\gamma$ 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 $\gamma$ (PPAR$\gamma$). However, the detailed mechanism of how telmisartan acts on PPAR$\gamma$ 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 PPAR$\gamma$ using isothermal titration calorimetry (ITC) and surface plasmon resonance (SPR) system. Based on physicochemical data, we then reevaluated the metabolic 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 PPAR$\gamma$ as well as the highest cell permeability, telmisartan superbly activates PPAR$\gamma$ 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; Lamouner-Zepter et al., 2006). Angiotensin II type 1 (AT$_1$) 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 AT$_1$ 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, PPAR$\gamma$ controls a wide variety of genes involved in fuel storage in adipose tissue and insulin resistance. For example, PPAR$\gamma$ activation facilitates the transcription of genes involved in lipid metabolism, including fatty acid activation and beta-oxidation (Varady et al., 2001; Bucala et al., 2001; Wisse et al., 2002). In addition, PPAR$\gamma$ is involved in the control of glucose homeostasis, acting as a critical mediator of adipose tissue dysfunction (Schenk et al., 2002; Tontonoz et al., 1994). Paradoxically, PPAR$\gamma$ expression is downregulated in obese tissues (Pfeffer et al., 1998), and the ratio of PPAR$\gamma$ to peroxisome proliferator-activated receptor-a (PPAR$\alpha$) expression is decreased in human and rat adipose tissue (Kadowaki et al., 2000). This downregulation may contribute to the development of obesity-related insulin resistance, as PPAR$\gamma$ activation improves insulin resistance and sensitizes adipocytes to insulin (Kim et al., 2002).

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ABBREVIATIONS: Ang II, angiotensin II; aP2, adipocyte fatty acid-binding protein; ARB, angiotensin II type 1 receptor blocker; AT$_1$, 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-1$\alpha$, PPAR$\gamma$-coactivator-1$\alpha$; PPAR$\gamma$, peroxisome proliferator-activated receptor $\gamma$; 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.

Telmisarten, 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, telmisarten 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 (SPPARM) (Schupp et al., 2005).

To clarify the mechanism by which telmisarten 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 physicochemical 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)methyl]phenyl] benzoic acid] was purchased from Nippon Boehringer Ingelheim Pharmaceuticals (Tokyo, Japan), and irbesartan [2-butyl-3-(4-[2-2H-1,2,3,4-tetrazol-5-yl]phenyl)(methyl)-1,3-diazaspiro[4.4](non-1-ene) was obtained from Shionogi Pharmaceutical (Tokyo, Japan). These compounds were then extracted by Astellas Pharmaceutical (Ibaraki, Japan). Azilsartan [2-ethoxy-1-(2-[5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl]biphenyl-4-yl)methyl]-1H-benzimidazole-7-carboxylic acid] was synthesized by Astellas Pharmaceutical as previously reported elsewhere (Kohara et al., 1996). Candesartan [2-ethoxy-1-(4-[2-2H-1,2,3,4-tetrazol-5-yl]phenyl)[phenyl](methyl)-1H-1,3-benzodiazole-7-carboxylic acid] was purchased from Sequoia Research Products (Pangbourne, UK). A thiazolidinedione derivative, pioglitazone ([R]-5-[1-3,4-ethylpyridin-2-yl)ethoxbenzyl]thiazolidine-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-methlyxanthine (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 (Merk Millipore, Darmstadt, Germany). HisTag-PPARγ LBD was expressed in Escherichia coli BL21(DE3) (Merk 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) (Merk 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 pACYC184 harboring the birA gene. In vivo biotinylation of PPARγ LBD was performed through expression. The target protein was purified with a nickel-nitrilotriacetic 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 syringe were prepared in the same buffer, which consisted of PBS, pH 7.4, with 5% DMSO. Binding affinities (Kd = 1/Ka) 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 Z7260-107; 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 LC1100; Agilent Technologies, Palo Alto, CA).

A parallel artificial membrane permeability assay (PAMPA) was used to assess cell permeability in a series of ARBs (Avdeef, 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 transactivation ability of ARBs (Benson et al., 2004; Schupp et al., 2011). Therefore, we assessed the lipophilicity and cell permeability ARBs tested. Telmisartan exhibited the highest affinity for PPARγ among various Types of ARBs Tested. The affinity (K_{d} = 1/K_{a}) 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 ($K_{d} = 340 \pm 40$ nM). In contrast, irbesartan showed marginal binding affinity for PPARγ ($K_{d} = 10.3 \pm 1.5$ 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; Kajiyama 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).
Lipid Accumulation in 3T3-L1 Adipocytes. To investigate the potential impact of each ARB on adipogenesis, 3T3-L1 preadipocytes were differentiated with each ARB, with replenishment of standard inducers of differentiation (i.e., IBMX, DEX, and insulin) (Frost and Lane, 1985; Ishii-Yonemoto et al., 2010). As shown in Fig. 1, telmisartan robustly and dose-dependently facilitated adipose differentiation at a concentration of 0.3 μM in 3T3-L1 cells. In contrast, only a higher dose (10 μM) of irbesartan slightly induced adipose differentiation. Likewise, a higher dose (10 μM) of azilsartan or candesartan minutely provoked adipose differentiation. However, the impact was marginal compared with telmisartan. These results are consistent with those of previous reports (Benson et al., 2004; Fujimoto et al., 2004; Schupp et al., 2004; Kajiya et al., 2011).

Effect of ARBs on αP2 and PPARγ2 mRNA in Cultured Adipocytes. To investigate the potential regulation of PPARγ target genes by ARBs in adipocytes, compounds were replenished with differentiation media from days 0 to 6. By addition of 10 μM telmisartan, the mRNA expression level of αP2 was markedly increased by approximately 3.4-fold compared with vehicle (DMSO) (Fig. 2A). In contrast, other ARBs did not increase the mRNA expression level of αP2. Further, telmisartan dose-dependently increased the mRNA expression level of PPARγ2 (Fig. 2B, 3.0-fold increase at 10 μM). Interestingly, a higher dose of irbesartan significantly increased the expression of PPARγ2 (3.3-fold increase at 10 μM). Azilsartan also significantly increased the expression of PPARγ2 (2.0-fold increase at 10 μM). However, it is of note that these effects were not dose-dependent. In contrast, candesartan did not increase the expression of PPARγ2 at all.

Effect of ARBs on 2-Deoxyglucose Transport in 3T3 Adipocytes. To evaluate the potential impact of ARBs on glucose transport in 3T3-L1 adipocytes, a 2-deoxyglucose (2-DG) uptake assay was conducted. As shown in Fig. 3, even in the absence of insulin, 1 and 10 μM telmisartan significantly augmented 2-DG uptake in adipocytes by 1.6- and 2.4-fold, respectively. In the presence of insulin, 1 and 10 μM telmisartan augmented 2-DG uptake in adipocytes by 1.8- and 2.7-fold, respectively. Likewise, in the absence or presence of insulin, 10 μM irbesartan augmented 2-DG uptake by 2.3- and 2.1-fold, respectively. In contrast, azilsartan at doses up to 10 μM did not affect 2-DG uptake. Although a higher dose (10 μM) of candesartan increased 2-DG uptake, the effect was marginal compared with telmisartan or irbesartan.

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 μM significantly increased GLUT4 protein by 3.0-fold. Likewise, irbesartan at 10 μM significantly increased GLUT4 protein expression by 2.8-fold. In contrast, azilsartan and candesartan did not increase GLUT4 protein expression.

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 (Kd) 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-planer conformation, might fit into the narrow, flat pocket of PPAR\(_g\). Second, the lipophilic property of benzimidazole rings and the pocket surface of PPAR\(_g\) 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\(_g\) agonism (Amano et al., 2012). In contrast, other ARBs would be unable to bind PPAR\(_g\) and form a hydrogen bond with Tyr473, in a similar fashion to telmisartan. Eprosartan, irbesartan, losartan, 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\(_g\) 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\(_g\) 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 for 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 activity of PPARγ without recruitment of TIF-2 (Schupp et al., 2005). 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

**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-pyridinyl-benzamide), 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.,

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**Fig. 5.** Unique structure of telmisartan. (A) Chemical structure of telmisartan divided into the A-part, which contains biphenyl carboxylic acid, and the B-part, which contains two benzimidazole rings. The central benzimidazole ring of the B-part is shown in green. (B) Top view of telmisartan bound to PPARγ. The structure is available in Protein Data Bank (3VN2). Telmisartan is shown in a space-filling model. Gray atoms indicate carbon atoms of the A-part. Yellow and green atoms indicate carbon atoms of the central benzimidazole ring and other regions of the B-part, respectively. Cyan atoms indicate carbon atoms of PPARγ. Red and blue atoms indicate oxygen and nitrogen atoms, respectively. Tyr473 makes a hydrogen bond with telmisartan. (C) Side view of the B-part in the hydrophobic and narrow pocket of PPARγ. A cross-sectional view is shown from the direction indicated by the arrow in (B). In this view, the A-part is removed to make it easier to recognize the B-part. Outset: Chemical structures of typical ARBs are shown around the inset. Sections corresponding to the central benzimidazole ring of telmisartan are shown in green.
In our report, the effect of telmisartan was examined in both “differentiating” adipocytes and “differentiated” adipocytes using 3T3-L1 cellular systems. Notably, telmisartan did increase the aP2 mRNA level at lower doses in differentiating adipocytes compared with differentiated adipocytes. On the basis of our previous findings, in our present study we examined the effect of telmisartan in differentiating adipocytes. Therefore, it is likely to speculate that telmisartan would increase the phosphorylation of insulin receptor, insulin receptor substrate-1, and protein kinase B at lower doses (1 μM or lower) in the differentiating 3T3-L1 adipocytes than in Furukawa’s differentiated 3T3-L1 adipocytes.

On the other hand, Lakshmanan et al. (2011) reported that telmisartan attenuates oxidative stress and rescues the down-regulation of Ang-(1–7) mas receptor protein in murine kidney. In the same report, they noted that telmisartan rescued the down-regulation of PGC-1α protein in murine kidney, thus suggesting that telmisartan might increase the expression of PPARγ and its downstream, PGC-1α, through the up-regulation of Ang-(1–7) mas receptor. Therefore, there is a possibility that the rescue of the down-regulation of the Ang-(1–7) mas receptor by telmisartan might also contribute to its metabolically beneficial effects.

Collectively, our data support the notion that telmisartan exerts a variety of metabolically beneficial effects within clinically available oral doses.

Conclusion

In this study of ARBs tested in a series of advanced analytic assays, telmisartan exhibited the highest cell permeability, the highest binding affinity, and the highest agonistic activities for PPARγ. The concentration of ARBs required for the augmentation of glucose uptake in cultured adipocytes is achieved only in the plasma of hypertensive patients treated with telmisartan. Such effects were not observed in irbesartan, azilsartan, or candesartan. In this context, among clinically 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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