FEBS Letters 576 (2004) 492–497 FEBS 28859

An angiotensin II AT_1 receptor antagonist, telmisartan augments glucose uptake and GLUT4 protein expression in 3T3-L1 adipocytes

Muneya Fujimoto[a], Hiroaki Masuzaki[a,*], Tomohiro Tanaka[a], Shintaro Yasue[a], Tsutomu Tomita[a], Kayoko Okazawa[a], Junji Fujikura[a], Hideki Chusho[a], Ken Ebihara[a], Tatsuya Hayashi[a,b], Kiminori Hosoda[a], Kazuwa Nakao[a]

[a]*Division of Endocrinology and Metabolism, Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine, 54, Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan*
[b]*Kyoto University Graduate School of Human and Environmental Studies, Japan*

Received 7 September 2004; revised 13 September 2004; accepted 13 September 2004

Available online 25 September 2004

Edited by Barry Halliwell

Abstract Evidence has accumulated that some of the angiotensin II AT_1 receptor antagonists have insulin-sensitizing property. We thus examined the effect of telmisartan on insulin action using 3T3-L1 adipocytes. With standard differentiation inducers, a higher dose of telmisartan effectively facilitated differentiation of 3T3-L1 preadipocytes. Treatment of both differentiating adipocytes and fully differentiated adipocytes with telmisartan caused a dose-dependent increase in mRNA levels for PPARγ target genes such as aP2 and adiponectin. By contrast, telmisartan attenuated 11β-hydroxysteroid dehydrogenase type 1 mRNA level in differentiated adipocytes. Of note, we demonstrated for the first time that telmisartan augmented GLUT4 protein expression and 2-deoxy glucose uptake both in basal and insulin-stimulated state of adipocytes, which may contribute, at least partly, to its insulin-sensitizing ability.

Keywords: Telmisartan; Angiotensin II AT_1 receptor antagonist; Peroxisome proliferator-activated receptor γ; Adipocyte; Glucose uptake; GLUT4

1. Introduction

Functional abnormalities in adipocytes have been implicated in the pathophysiology of type 2 diabetes and the metabolic syndrome [1]. Peroxisome proliferator-activated receptor γ (PPARγ), a nuclear receptor abundantly expressed in adipocytes, plays a pivotal role in adipocyte differentiation, function and distribution through regulating a wide variety of target genes [2]. Actually, synthetic ligands for PPARγ, thiazolidinedione derivatives (TZDs), have proven to be effective for the treatment of insulin resistance and diabetes in humans [3,4]. Although TZDs such as pioglitazone [5] and rosiglitazone [6] are widely employed in clinical practice, in some cases, these compounds cause adverse effects such as fluid retention, peripheral edema and heart failure [7].

A number of clinical trials have suggested that angiotensin-converting enzyme inhibitors or angiotensin II AT_1 receptor antagonists (or angiotensin II receptor blockers, ARBs) can improve insulin sensitivity and significantly reduce the incidence of newly occurred type 2 diabetes in patients with hypertension [8,9]. However, the mechanism whereby blockade of renin–angiotensin system enhances whole body insulin sensitivity has not been fully elucidated so far [10,11]. Very recently, it has been reported that a clinically used ARB, telmisartan, shares a structural similarity with pioglitazone and can serve as a partial agonist of PPARγ [12,13]. The work demonstrated that telmisartan uniquely augmented the expression of established PPARγ target genes including aP2 (FABP4), CD36, and acetyl coenzyme A carboxylase (ACC) in murine and human adipocytes and murine myocytes [12] as well as induced adipogenesis in 3T3-L1 preadipocytes [12]. Furthermore, data have shown that orally-administered telmisartan improved glucose and lipid homeostasis in rats fed high-fat and high-carbohydrate diet [12]. However, underlying mechanism responsible for insulin-sensitizing effects by telmisartan has not been fully addressed. Moreover, systemic administration experiments would not be enough to clarify the exact action site of telmisartan in terms of improvement of fuel homeostasis.

In this context, the present study was designed to elucidate the effect of telmisartan on insulin action using 3T3-L1 adipocytes. We here show that telmisartan coordinately regulates mRNA expression of insulin-sensitizing hormone, adiponectin [14,15] and insulin resistance-inducing enzyme, 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) [16,17], which may favor adipocytes with insulin sensitization. We also demonstrate for the first time that telmisartan augments GLUT4 protein expression and 2-deoxy glucose uptake in 3T3-L1 adipocytes, providing evidence that telmisartan exerts its insulin-sensitizing effects directly on adipocytes.

2. Materials and methods

2.1. Materials

Angiotensin II AT_1 receptor antagonists, telmisartan (BIBR 277) and valsartan (CGP 48933), and a thiazolidinedione derivative, pioglitazone (AD-4833), were generously provided from Nippon Boehringer Ingelheim (Tokyo, Japan), Novartis Pharma (Tokyo, Japan) and Takeda Chemical Industries (Osaka, Japan), respectively. Human recombinant insulin was purchased from Roche Diagnostics (Tokyo, Japan). 3-Isobutyl-1-methylxanthine (IB) and dexamethasone (DX)

* Corresponding author. Fax: +81-75-771-9452.
E-mail address: hiroaki@kuhp.kyoto-u.ac.jp (H. Masuzaki).

0014-5793/$22.00
doi:10.1016/j.febslet.2004.09.027

were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Dulbecco's Modified Eagle's Medium (DMEM), Calf Serum (CS) and Fetal Bovine Serum (FBS) were from Invitrogen Corp. (Carlsbad, CA, USA). ECL plus western detecting kit was purchased from Amersham Biosciences Corp. (Piscataway, NJ, USA).

2.2. Cell culture and Oil Red O staining

3T3-L1 cells (kindly provided by Dr. H. Green and Dr. M. Morikawa, Harvard Medical School, Boston, MA, USA) were cultured and differentiated into adipocytes as described previously [18]. Briefly, cells were grown for 2 days post-confluence (referred as day 0) in 10% CS/DMEM. Differentiation was induced with 10% FBS/DMEM containing 0.5 mM IB, 0.25 μM DX and 1 μg/ml insulin for 2 days. The cells were then incubated in 10% FBS/DMEM with insulin for 2 days and maintained hereafter with 10% FBS/DMEM to day 8. Telmisartan, valsartan and pioglitazone were dissolved in DMSO and added to media within 0.1% of volume. Medium was changed every other day. At day 8, the cells were washed with PBS twice, fixed in 3.7% formaldehyde for 1 h and then stained with 0.6% (w/v) Oil Red O solution (60% isopropanol, 40% water) for 2 h at room temperature. Cells were then washed with water to remove unbound dye. Stained Oil Red O was eluted with isopropanol and quantified by measuring the optical absorbance at 510 nm [19].

2.3. Northern blot analyses and quantitative real time PCR

During the course of adipose differentiation, all compounds including DMSO (vehicle) were added to media on day 2 (differentiating state) or on day 8 (mature adipocyte) for 24 h. Although insulin was added to media from day 2 to day 3, media without insulin were used in case of extracting RNA on day 3. After 24 h incubation, total RNA of 3T3-L1 cells was prepared using Trizol Reagent (Invitrogen). To determine mouse aP2 and adiponectin mRNA expression levels, equal amount of total RNA (20 μg/each lane) was electrophorased, transferred and Northern blot hybridization was performed as described previously [20]. For quantitative RT-PCR assay, cDNA was prepared from total RNA using SuperScript First-Strand Synthesis System (Invitrogen). To determine 11β-HSD1 mRNA levels, these probe and primers were employed [21]: probe (5′FAM-cgtcatctcctccttggctgggaa-TAMRA-3′), forward (5′-agcagagcaatggcagcat-3′) and reverse (5′-gagcaatcataggctgggtca-3′). TaqMan PCR was performed using ABI Prism 7700 Sequence Detection System as instructed by manufacturer (Applied Biosystems, Foster City, CA, USA). Results were normalized to endogenous control 18S mRNA concentrations.

2.4. Western blot analyses

Cells were washed twice with ice-cold PBS and harvested in a lysis buffer (10 mM Tris–HCl, 1 mM EDTA, 255 mM sucrose, 1% (v/v) Nonidet P-40, 1 mM PMSF and 0.1 mg/ml aprotinin at pH 7.4). After centrifugation, supernatants were normalized for protein concentration via Bradford method and subjected to immunoblotting. Western blot analyses were performed using anti-polyclonal GLUT4 antibody (Santa Cruz Biotechnology, California, CA, USA) as reported previously [22].

2.5. 2-Deoxy glucose uptake assay

Cells were treated with 0.1% DMSO, telmisartan, valsartan or pioglitazone throughout the course of differentiation. On day 8, glucose transport by monolayer of 3T3-Ll adipocytes was assessed by the uptake of 2-deoxy-L-[^{3}H]glucose (Perkin Elmer Life Sciences, Boston, MA, USA) as reported previously [20].

2.6. Statistical analyses

The data are presented as means ± S.E.M. Student's t test was used to compare the data between the vehicle (DMSO) and treated group. Differences were accepted as significant at $P < 0.05$ level.

3. Results

3.1. Telmisartan facilitates lipid accumulation in 3T3-L1 adipocytes

To explore the potential effect of telmisartan on adipogenesis, 3T3-L1 preadipocytes were differentiated with telmisartan in addition to standard differentiation inducers (i.e., IB, DX and insulin) [18]. As shown in Fig. 1, higher doses (1 and 10 μM) of telmisartan facilitated differentiation of 3T3-L1 cells, whereas a lower dose (0.1 μM) did not. On the contrary, valsartan had no effect on adipogenesis even with a concentration of 10 μM. These data were well in agreement with previous report [12]. Also, consistent with previous reports [23,24], pioglitazone facilitated the differentiation of preadipocytes with a concentration of 1 μM.

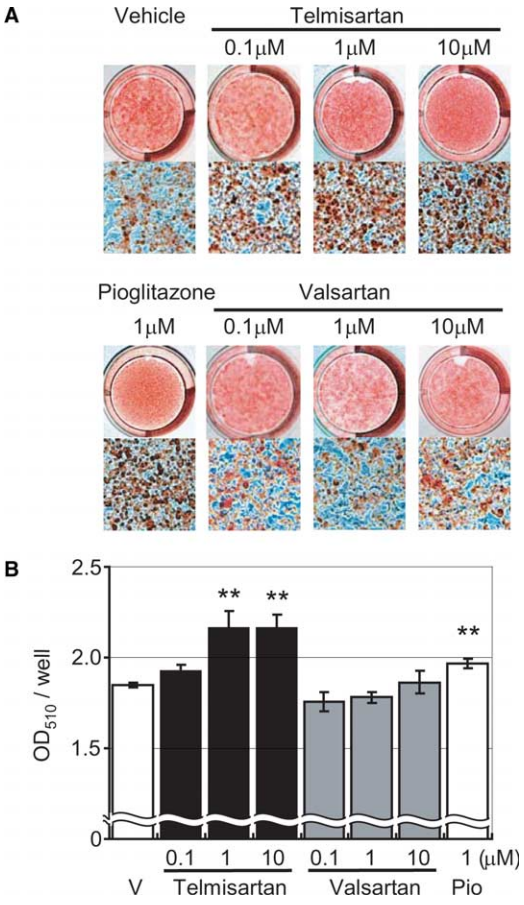

Fig. 1. Effect of telmisartan on adipose differentiation of 3T3-L1 cells. Telmisartan, valsartan and pioglitazone were dissolved in DMSO (vehicle) and added to media from the start of differentiation (day 0) to mature adipocyte (day 8). (A) Differentiated adipocytes were fixed and stained with Oil Red O at day 8. Macroscopic and microscopic pictures (magnitude 50×) of cells are shown. (B) Lipid accumulation was assessed by the quantification of OD_{510} in destained Oil Red O with isopropanol. Telmisartan significantly facilitated adipogenesis in a concentration of 1 μM (17 ± 6% increase) and 10 μM (17 ± 5% increase). Data are expressed as means ± S.E.M. from triplicate experiments. $^{**}P < 0.01$ (Student's t test) compared with vehicle (V) treated group. Pio: pioglitazone.

3.2. Telmisartan regulates a line of PPARγ target genes

To elucidate regulation of PPARγ downstream genes by telmisartan in adipocytes, compounds were added to differentiation media on day 2 (Fig. 2) or on day 8 (Fig. 3) for 24 h. By addition of 10 μM telmisartan from day 2 to day 3 (differentiating state), mRNA levels of aP2 and adiponectin were increased about 2.4 ± 0.1-fold ($P < 0.01$) and 2.2 ± 0.1-fold ($P < 0.01$) compared to vehicle (DMSO) treatment, respectively (Fig. 2A and B). Noteworthy is that such an increase in

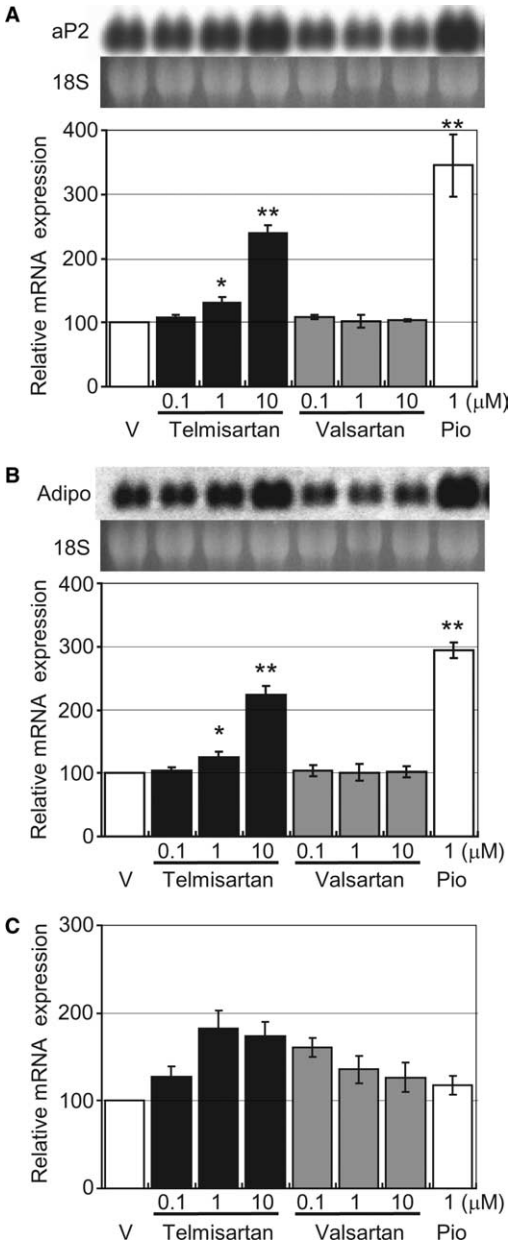

Fig. 2. Telmisartan regulates PPARγ target genes in differentiating 3T3-L1 cells (day 3). Cells were differentiated and treated with compounds on day 3 for 24 h. Total RNA was extracted and Northern blot analyses of aP2 (A) and adiponectin (B) were performed. Quantification of relative mRNA expression was shown. (C) Specific mRNA for 11β-HSD1 was determined by quantitative RT-PCR. Results were normalized to the signal generated from 18S mRNA. Data are expressed as means ± S.E.M. from quadruplicate (aP2 and adiponectin) or triplicate (11β-HSD1) experiments. $*P < 0.05$, $**P < 0.01$ (Student's t test) compared with vehicle (V) treated group. Adipo: adiponectin, Pio: pioglitazone.

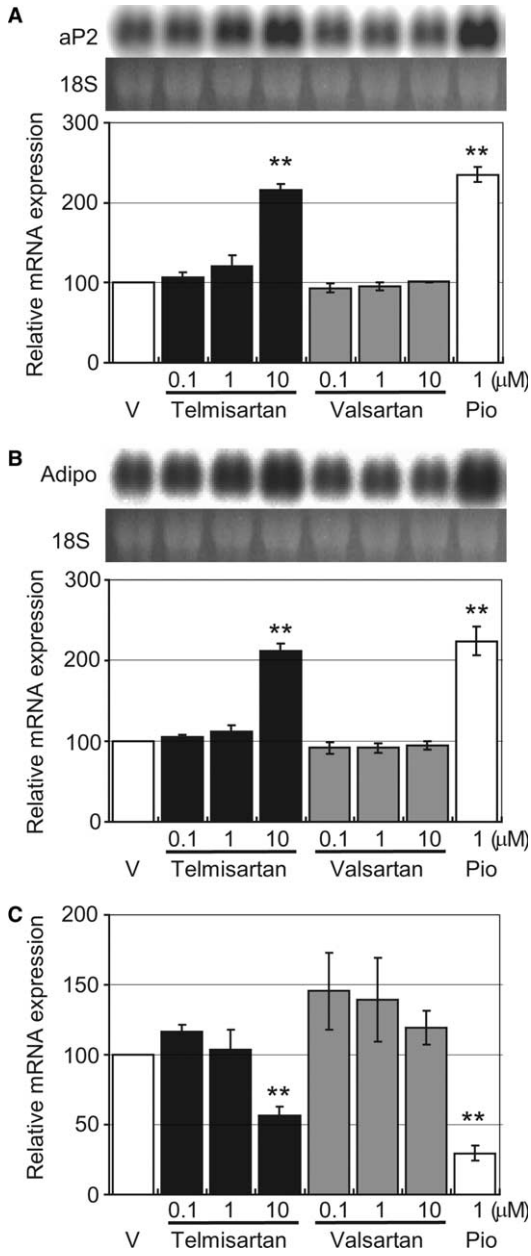

Fig. 3. Telmisartan regulates PPARγ target genes in differentiated 3T3-L1 adipocytes (day 9). Cells were differentiated to mature adipocyte (day 8) and treated with compounds for 24 h. Northern blot analyses of aP2 (A) and adiponectin (B) were performed. Quantification of relative mRNA expression was shown. (C) Specific mRNA for 11β-HSD1 was determined by quantitative RT-PCR. Results were normalized to the signal generated from 18S mRNA. Data are expressed as means ± S.E.M. from quadruplicate (aP2 and adiponectin) or triplicate (11β-HSD1) experiments. $**P < 0.01$ (Student's t test) compared with vehicle (V) treated group. Adipo: adiponectin, Pio: pioglitazone.

mRNA level was in a dose-dependent manner. On the other hand, valsartan did not augment mRNA expression of aP2 and adiponectin. The level of mRNA for 11β-HSD1, which has been shown to increase by 500-fold during differentiation of 3T3-L1 cells [25], was not changed by telmisartan, valsartan or pioglitazone (Fig. 2C).

When ARBs were added to media after cells were differentiated into adipocytes, aP2 and adiponectin mRNA levels were also elevated only by 10 μM telmisartan (2.2 ± 0.1-fold ($P < 0.01$) and 2.1 ± 0.1-fold ($P < 0.01$), respectively), whereas valsartan did not exert such effects (Fig. 3A and B). In differentiated adipocytes, gene expression of 11β-HSD1, known to be downregulated by PPARγ ligand [25], was significantly decreased by 10 μM telmisartan (0.6 ± 0.1-fold ($P < 0.01$)), but not by valsartan (Fig. 3C).

3.3. Effect of telmisartan on 2-deoxy glucose transport in 3T3-L1 adipocytes

To examine a potential impact of telmisartan on glucose transport in differentiated 3T3-L1 adipocytes, 2-deoxy glucose (2-DG) uptake assay was performed. Although treatment with valsartan did not affect 2-DG uptake even with a concentration of 10 μM, telmisartan significantly increased glucose uptake. Even in the absence of insulin (Fig. 4A), 1 and 10 μM telmisartan significantly augmented 2-DG uptake in adipocytes by 1.9 ± 0.2-fold ($P < 0.05$) and 3.0 ± 0.2-fold ($P < 0.01$), respectively. In the presence of 1 μM insulin (Fig. 4B), 1 and 10 μM telmisartan also significantly augmented glucose uptake by 1.7 ± 0.2-fold ($P < 0.05$) and 2.7 ± 0.5-fold ($P < 0.01$), respectively.

3.4. Telmisartan increases GLUT4 protein level in 3T3-L1 adipocytes

Based on our result that telmisartan has a robust effect on glucose uptake, we next assessed its impact on GLUT4 protein expression. Western blot analyses showed that, when cells were differentiated with 1 or 10 μM of telmisartan, GLUT4 protein was significantly increased compared to DMSO treatment (1.4 ± 0.1-fold, or 1.3 ± 0.1-fold, $P < 0.05$, respectively) (Fig. 5). In contrast, valsartan did not augment GLUT4 protein expression even with a concentration of 10 μM.

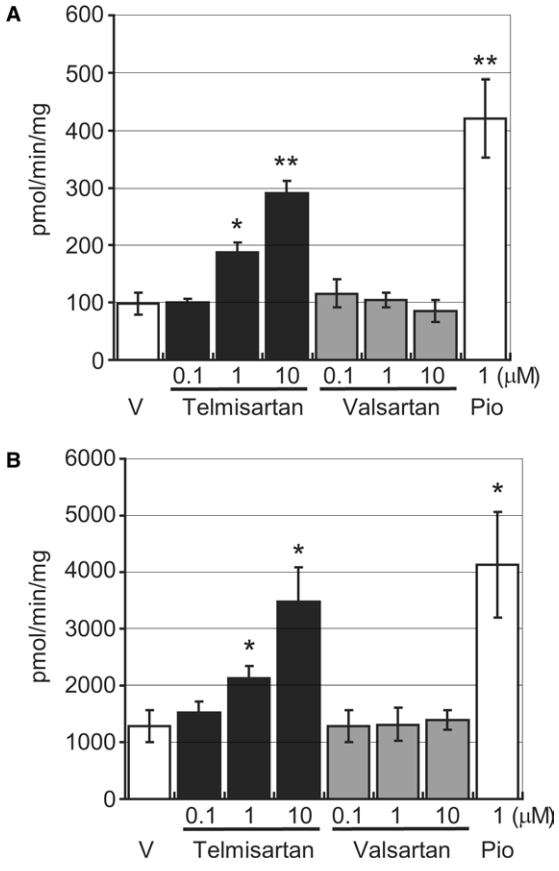

Fig. 4. Telmisartan increases glucose transport in 3T3-L1 adipocytes. Cells were treated with compounds throughout the course of adipocyte differentiation (day 0–day 8). 2-DG uptake was measured in the absence (A) or presence (B) of 1 μM insulin. Telmisartan significantly augmented 2-DG uptake in a dose-dependent manner. Data are presented as means ± S.E.M. from quadruplicate experiments. *$P < 0.05$, ** $P < 0.01$ (Student's t test) compared with vehicle (V) treated group. Pio: pioglitazone.

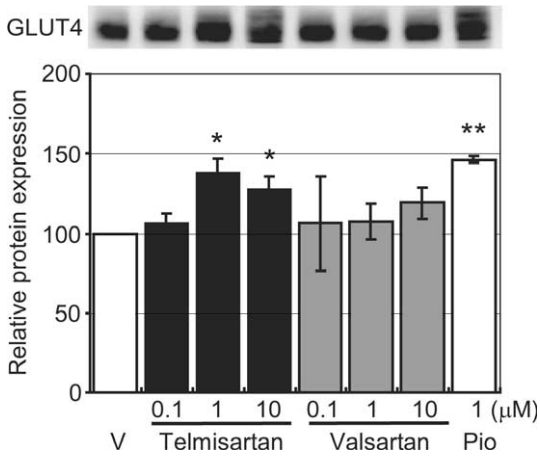

Fig. 5. Telmisartan significantly increases GLUT4 protein expression in 3T3-L1 adipocytes. Cells were treated with compounds throughout the course of differentiation. On day 8, total cell lysates were prepared and equal amount of protein (5 μg) was subjected to Western blotting. Quantification of relative protein expression was plotted. Data are presented as mean ± S.E.M. from triplicate experiments. *$P < 0.05$, **$P < 0.01$ (Student's t test) compared with vehicle (V) treated group. Pio: pioglitazone.

4. Discussion

We here demonstrate that telmisartan dose-dependently augments GLUT4 protein expression and 2-deoxy glucose uptake both in basal and insulin-stimulated state of 3T3-L1 adipocytes. The potency of such insulin-sensitizing effects is comparable to that seen in pioglitazone-treated cells and is not observed in cells treated with valsartan. To the best of our knowledge, this is the first report providing evidence that telmisartan exerts its insulin-sensitizing effects directly on adipocytes. Consistent with our data, previous reports have shown that TZDs augment GLUT4 protein expression and glucose uptake in cultured adipocytes [23,24,26,27]. Although several mechanisms are involved in the molecular control of GLUT4 expression by TZDs [28–30], the result allows us to speculate that telmisartan induces GLUT4 expression and subsequent glucose uptake, at least in part, via PPARγ activation in adipocytes.

A line of evidence has suggested that the activation of the renin–angiotensin system impairs early steps of insulin receptor signaling such as tyrosine phosphorylation of insulin receptor substrate 1 or activation of PI3-kinase both in vivo and in vitro [31,32]. On the other hand, it has been shown that chronic angiotensin II receptor antagonism via clinically employed ARB, irbesartan increases muscle GLUT4 protein levels in Zucker fatty (fa/fa) rats [33]. Taken together, it is tempting to speculate that the blockade of angiotensin II receptor signaling might exert metabolically beneficial effects in adipocytes. Nevertheless in skeletal muscle of type 2 diabetic mouse, valsartan has been shown to enhance GLUT4 translocation to plasma membrane [34], no reports have shown that ARBs increase GLUT4 protein expression and subsequent

glucose uptake in adipocytes. In this context, our data provide evidence that telmisartan is the case.

It has been widely recognized that PPARγ expressing in "extra-adipose" tissues such as macrophage, colon, liver, skeletal muscle, pancreatic β cells or pituitary cells is also involved in a line of pathologic conditions including type 2 diabetes, steatosis, atherosclerosis and cancer [35,36]. Moreover, angiotensin II AT_1 receptor has been shown to express in a wide variety of tissues including adipocytes [37–39]. Thus, an entire picture of the in vivo mechanism whereby telmisartan improves glucose homeostasis must await further investigation. However, our results suggest that adipocytes are one of the major sites of action for telmisartan in terms of fuel metabolism.

A growing body of evidence has suggested that PPARγ agonists have the strong potential to correct functional abnormalities in adipocytes including amelioration of dysregulation in adipocytokine release (see [1] for review). A prototype of adipocytokines, adiponectin, has been recognized as a fat-cell derived insulin-sensitizing hormone [14,15]. Our data demonstrate that, in both differentiating and differentiated 3T3-L1 adipocytes, telmisartan augments mRNA expression of adiponectin in a dose-dependent manner, which may also contribute to metabolically-beneficial effects of telmisartan.

Furthermore, the present study demonstrates that, in differentiated adipocytes, telmisartan substantially decreases mRNA level for 11β-HSD1, an intracellular glucocorticoid reactivating enzyme [16,17]. Evidence has accumulated that locally-enhanced action of glucocorticoid in adipocytes, mediated by exaggerated activation of 11β-HSD1, plays an important role in adipocyte dysfunction and adipocytokine dysregulation [16,17,40,41]. Taken together, it is reasonable to speculate that telmisartan-induced decrease in the mRNA level for 11β-HSD1 favors adipocytes with insulin sensitization. It is known that gene expression of 11β-HSD1 is markedly induced (∼ by 500-fold) during the course of adipose differentiation [25], which may explain, at least in part, why we failed to observe the decrease in mRNA level for 11β-HSD1 by telmisartan in differentiating adipocytes.

In summary, the present study first provides evidence that telmisartan enhances glucose uptake in cultured adipocytes, accompanied by an increase in GLUT4 expression. Coordinated regulation of mRNA expressions for adiponectin and 11β-HSD1 in adipocytes may also be beneficial for insulin-sensitizing effects by telmisartan. Our data provide a fresh insight into improved therapeutic approaches to treat type 2 diabetes, hypertension with insulin resistance and the metabolic syndrome.

Acknowledgements: We thank Ms. M. Tsuchiya and Ms. S. Shinhara for secretarial assistance. We also thank Ms. T. Ishii, Ms. M. Nagamoto, Ms. Z. Lanying and Mr. N. Arai for technical help. This work is supported by Grant-in-Aid for Scientific Research (B2) (16390267), Grant-in-Aid for Scientific Research (S2) (16109007), Grant-in-Aid for Exploratory Research (16659243), Grant-in-Aid for Scientific Research on Priority Areas (15081101), The Ministry of Education, Culture, Sports, Science and Technology of Japan, Research Grant from Special Coordination Funds for Promoting Science and Technology, Research award from Japan Foundation for Applied Enzymology, Tanita Healthy Weight Community Trust, Daiwa Securities Health Foundation, ONO Medical Research Foundation, Yamaguchi Endocrine Research Association, The Ichiro Kanehara Foundation, Yamanouchi Foundation for Research on Metabolic Disorders, The Cell Science Research Foundation, Takeda Medical Research Foundation, The Study Grant for Japan Insulin Study Group, Smoking Research Foundation and the metabolic syndrome research foundation.

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