Telmisartan-Induced Inhibition of Vascular Cell Proliferation Beyond Angiotensin Receptor Blockade and Peroxisome Proliferator-Activated Receptor-γ Activation

Koichi Yamamoto, Mitsuru Ohishi, Christopher Ho, Theodore W. Kurtz, Hiromi Rakugi

Abstract—We investigated the ability of angiotensin II type 1 (AT1) receptor blockers with peroxisome proliferator-activated receptor (PPAR)-γ agonist activity (telmisartan and irbesartan) and AT1 receptor blockers devoid of PPAR-γ agonist activity (eprosartan and valsartan) to inhibit vascular cell proliferation studied in the absence of angiotensin II stimulation. Telmisartan and, to a lesser extent, irbesartan inhibited proliferation of human aortic vascular smooth muscle cells in a dose-dependent fashion, whereas eprosartan and valsartan did not. To investigate the role of PPAR-γ in the antiproliferative effects of telmisartan, we studied genetically engineered NIH3T3 cells that express PPAR-γ. Pioglitazone inhibited proliferation of NIH3T3 cells expressing PPAR-γ but had little effect on control NIH3T3 cells that lack PPAR-γ. In contrast, telmisartan inhibited proliferation equally in NIH3T3 with and without PPAR-γ. Valsartan failed to inhibit proliferation of either cell line. In addition, telmisartan inhibited proliferation equally in aortic smooth muscle cells derived from mice with targeted knockout of PPAR-γ in the smooth muscle and from control mice, whereas valsartan had no effect on cell proliferation. Telmisartan, but not valsartan, reduced phosphorylation of AKT but not extracellular signal–regulated kinase otherwise induced by exposure to serum of quiescent human smooth muscle cells, quiescent mice smooth muscle cells lacking PPAR-γ, or quiescent Chinese hamster ovary-K1 cells lacking the AT1 receptor. In summary, the antiproliferative effects of telmisartan in the absence of exogenously supplemented angiotensin II involve more than just AT1 receptor blockade and do not require activation of PPAR-γ. It might be postulated that inhibition of AKT activation is a mechanism mediating the antiproliferative effects of telmisartan, including in cells lacking AT1 receptors or PPAR-γ. (Hypertension. 2009;54:1353-1359.)

Key Words: atherosclerosis ■ angiotensin type II receptor blocker ■ cell proliferation ■ PPAR-γ ■ angiotensin II

Vascular proliferation is a critical process in the development of atherosclerosis and is associated with other cellular processes in atherogenesis, such as inflammation, apoptosis, and matrix formation.1 Many mediators are involved in the pathogenesis of vascular proliferation, including growth factors and cytokines, such as platelet-derived growth factor, transforming growth factor-β, angiotensin II (AII), epidermal growth factor, and insulin-like growth factor 1.1 Inhibition of AII-mediated vascular proliferation by angiotensin-converting enzyme inhibitors and AT1 type 1 (AT1) receptor blockers (ARBs) is considered a potential therapeutic mechanism for reducing the risk for atherosclerotic cardiovascular disease beyond just the ability of these drugs to reduce blood pressure.2 However, because vascular cell proliferation and atherosclerosis can be influenced by many growth factors other than just AT1, additional targets for inhibiting vascular cell growth beyond renin-angiotensin system blockade might be useful in maximizing cardiovascular protection.

Recently, certain ARBs, including telmisartan and irbesartan, have been reported to have partial peroxisome proliferator-activated receptor (PPAR)-γ-activating properties and have been referred to as selective PPAR modulators.3–5 Telmisartan has attracted particular attention because of its potential ability to activate PPAR-γ when tested at concentrations that might approach those achieved in plasma with the usual oral dosing. Telmisartan has been reported to increase adiponectin expression in adipocytes,6 inhibit migration of CD4-positive lymphocytes,7 delay endothelial cell senescence by regulating endothelial NO synthase production,8 and increase caloric expenditure and protect against dietary-induced weight gain and hepatic steatosis9 through mechanisms involving more than just AT1 receptor blockade and possibly via PPAR-γ activation. Recently, we reported that telmisartan can also inhibit vascular cell proliferation through mechanisms that do not appear to depend on the interaction of telmisartan with AT1 receptors.10 However, pretreatment of vascular cells with the PPAR-γ antagonist

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From the Department of Geriatric Medicine (K.Y., M.O., H.R.), Osaka University Graduate School of Medicine, Osaka, Japan; Department of Laboratory Medicine (K.Y., C.H., T.W.K.), University of California, San Francisco, San Francisco, Calif.
Correspondence to Koichi Yamamoto, Department of Geriatric Medicine, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan. E-mail kyamamoto@geriat.med.osaka-u.ac.jp
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GW9662 did not influence the antiproliferative effects of telmisartan, suggesting that the ability of telmisartan to inhibit cell proliferation might not involve activation of PPARγ.10 Because GW9662 itself can inhibit cell proliferation6,11 and because such pharmacological antagonists can have off-target effects or yield incomplete receptor inhibition, the studies with GW9662 left open the possibility that PPARγ activation might still be contributing to the antiproliferative effects of telmisartan. To definitively investigate whether PPARγ activation might be required for the antiproliferative effects of telmisartan, we investigated the ability of telmisartan to inhibit the proliferation of cell lines lacking PPARγ and explored the potential effects of telmisartan on selected signaling proteins involved in cell pathways that control growth and differentiation.

Materials and Methods

Materials
ARBs were purchased from the pharmacy, purified by high-performance liquid chromatography, and stored as stock solutions as dimethyl sulfoxide in the freezer.

Cell Culture
Human aortic vascular smooth muscle cells (HAVSMCs) and Chinese hamster ovary (CHO)-K1 cells were purchased from the American Type Culture Collection. HAVSMCs were maintained in DMEM/F12 supplemented with 10% FBS (Hyclone) and 1× Antibiotic-Antimycotic Solution (MediaTech, Inc), and CHO-K1 cells were maintained in DMEM supplemented with 10% FBS and 1× Antibiotic-Antimycotic Solution. Human coronary artery smooth muscle cells (HCASMCs) and human umbilical vein endothelial cells (HUVECs) were purchased from Lonza. HCASMCs were maintained in smooth muscle growth medium (Lonza), and HUVECs were maintained in endothelial growth medium 2 (Lonza). NIH3T3 cells stably transduced with the pBABE-Puro vector (NIH-Babe cells hereafter referred to as “NIH3T3 controls”) or the pBABE-Puro-PPARγ vector (NIH-Pparg cells hereafter referred to as “NIH3T3-Pparg cells”) were originally produced by Tomonozu et al12 and obtained from Dr Ron Evans (Salk Institute). NIH3T3 cells were maintained in DMEM supplemented with 5% calf serum (Hyclone), 2.0 mg/mL of BSA (Sigma), and 2.0 mg/mL of BSA. The aorta was incubated for 30 minutes. The medium was changed to DMEM that contained 1.0 mg/mL of trypsin inhibitor type I-S (Sigma), 1.0 mg/mL of trypsin inhibitor type I-S (Sigma), and 2.0 mg/mL of BSA. The aorta was incubated for 30 minutes. The cellular digests were filtered through sterile 100-μm nylon cell strainers, centrifuged at 300g for 10 minutes, and washed twice before culture in DMEM/F12 (University of California San Francisco Cell Culture Facility) containing 10% FBS (Hyclone) and antibiotics. All of the incubations in the enzyme solutions were carried out in 95% air/5% CO2 at 37°C. The study protocol was approved by the institutional animal care and use committee at the University of California San Francisco and was conducted in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Confirmation of Pparg Knockout in Smooth Muscle Cells
To confirm deletion of Pparg sequences flanked by loxP sites, RNA was extracted from vascular smooth muscle cells of SMPGKO and control mice using the RNAqueous-4PCR kit (Ambion). cDNA was synthesized using the SuperScript first-strand synthesis system (Invitrogen). Primers that distinguish the full-length (700-bp) and recombined (300-bp) transcripts of Pparg13 were used according to the previous report13 (forward primer in 5′ untranslated region: 5′-GTCACAGTCGTGACAGGACTGTGTGAC-3′; reverse primer in exon 4: 5′-TATCACTGGGAGATCTCCGCGCAACAGC-3′). Stepdown PCR was performed, and the products were electrophoretically separated on 7% polyacrylamide gel.

Cell Proliferation Assay
HAVSMCs, HUVECs, HCASMCs, NIH3T3 cells, and mouse aortic vascular smooth muscle cells were plated at 1×104 to 5×104 cells per well in the indicated medium. After attachment, cells were exposed to ARBs dissolved in DMSO, which were added in the same growth medium. Untreated cells not exposed to ARBs were incubated with an equivalent volume of DMSO added in the medium. Cells were treated with the indicated drugs for 5 to 7 days, and the medium containing drugs was changed twice. After 5 to 7 days, cells were trypsinized and cell numbers were counted using a hemacytometer.

Detection of Cell Signaling Proteins
We used Western blot analysis to detect effects of telmisartan or valsartan on serum-induced changes in phospho-AKT and phospho-p44/42 mitogen-activated protein kinase (extracellular signal–regulated kinase [ERK] 1 and ERK2) levels in vascular smooth muscle. Cells were made quiescent with low serum medium (0.25% FBS) or medium without serum, including telmisartan, valsartan, or DMSO vehicle for 24 hours, and were then changed to medium with 10% FBS. After 10 minutes, cells were washed twice with cold PBS and lysed with cell lysis buffer (Thermo Scientific) supplemented with complete mini-protease inhibitor mixture (Roche). After a centrifugation at 15 000g for 10 minutes, protein concentration was measured with the DC Protein assay kit (BioRad). Twenty-microgram aliquots of protein were separated by polyacrylamide-10% SDS gel electrophoresis and transferred to nitrocellulose membranes (Amer sham). After blocking with 5% skim milk, membranes were incubated with either phospho-AKT antibody that detects endogenous levels of AKT phosphorylated at Thr308 (Cell Signaling Technology, No. 5106) or phospho-ERK antibody that detects endogenous levels of p44 and p42 mitogen-activated protein kinase (ERK1 and ERK2) antibodies were either individually or dually at Thr202 and Tyr204 for ERK1 or Thr185 and Tyr187 for ERK2 (Cell Signaling Technology, No. 9101). This was followed by incubation with either antimouse secondary antibody or antirabbit secondary antibody, respectively. Chemiluminescent signals were generated by ECL-plus (Amer sham). After removal of the antibodies using the Restore buffer (Pierce), the membranes were subjected to immunodetection of total AKT using AKT antibody (Cell Signaling Technology, No. 9272) or total ERK using ERK antibody (Cell Signaling Technology, No. 9102). Signal intensities of the bands were analyzed by Multi Gauge software (Fuji Film), and the ratio of the signal intensity of phospho-AKT or phospho-ERK to that of total AKT or total ERK was calculated to determine the relative amounts of phospho-AKT or phospho-ERK, respectively.

Statistical Analysis
Data are expressed as mean±SEM. To compare cell numbers, statistical analysis was performed by 1-way ANOVA and the Holm-Sidak procedure to adjust for multiple comparisons of individual data groups against the control.
Results

Effect of ARBs on Cell Proliferation in Human Vascular Cells

We compared the effects of telmisartan, irbesartan, valsartan, and eprosartan on the spontaneous growth of human vascular cell lines under conditions in which the cells had not been supplemented with exogenous AII to stimulate proliferation. Three cell lines (HAVSMCs, HUVECs, and HCASMCs) were treated with different ARBs at a concentration of 10 μmol/L for 7 days and the cell numbers were counted. Telmisartan inhibited cell proliferation in all 3 of the cell lines, whereas the other ARBs had no effect on cell numbers (Figure 1). To test for dose-dependent effects of ARBs on cell proliferation, HAVSMCs were exposed to telmisartan, irbesartan, valsartan, or eprosartan at concentrations of 0.625, 1.250, 2.500, 5.000, 10.000, 20.000, 40.000, or 80.000 μmol/L for 7 days. Telmisartan and irbesartan inhibited cell proliferation in a dose-dependent fashion, whereas valsartan and eprosartan did not. Results are expressed as the percentage of cell numbers in control cells not treated with ARBs. Experiments were performed in triplicate. \( ^*P < 0.01 \) vs control.

Effect of Telmisartan on Cell Proliferation in NIH3T3 Cells Expressing or Not Expressing PPARγ

To determine whether the antiproliferative effects of telmisartan depend on PPARγ, we studied NIH3T3 cells lacking PPARγ and NIH3T3 cells stably expressing PPARγ. Figure 4 shows the effects of telmisartan and valsartan on proliferation of NIH3T3-PPARγ cells and NIH3T3-control cells in continuous culture (Figure 3). Experiments were also performed in cells treated with the PPARγ agonist pioglitazone as a positive control. Antiproliferative effects of pioglitazone were significantly greater in NIH3T3 cells expressing PPARγ compared with NIH3T3 cells lacking PPARγ. In contrast, the
supplementary amounts of AII. Two of the ARBs tested, telmisartan, have little or no effect on PPARγ and irbesartan, are known to have partial agonist activity on PPARγ. These findings raised the possibility that telmisartan might also have the ability to inhibit the proliferation of vascular smooth muscle cells in the absence of PPARγ.

Effect of Telmisartan on Cell Proliferation in PPARγ-Deleted Vascular Smooth Muscle Cells

To directly determine whether the antiproliferative effect of telmisartan in vascular smooth muscle cells requires PPARγ, we studied aortic vascular smooth muscle cells from SMPGKO mice and their littermate controls. Successful deletion of exons 1 and 2 of PPARγ in the SMPGKO mice was confirmed by RT-PCR (Figure 4). Pioglitazone (10 µM) significantly inhibited proliferation of vascular smooth muscle cells isolated from control mice but not in cells isolated from SMPGKO mice (Figure 5). Valsartan failed to inhibit proliferation in both knockout and control cells. However, 10 µM/L and 20 µM/L of telmisartan inhibited proliferation equally in cells isolated from SMPGKO mice and controls (Figure 5).

Effect of Telmisartan on Phosphorylation of AKT and ERK

Activation of AKT and ERK signaling by phosphorylation plays a key role in cell proliferation in response to growth stimuli. Therefore, we investigated the effects of different ARBs on the phosphorylation of AKT and ERK in HAVSMCs stimulated with 10% serum for 10 minutes after 24-hour cellular quiescence. As shown in Figure 6, the relative amount of AKT phosphorylated at Thr 308 was decreased by treatment with telmisartan beginning at concentrations of 2.5 to 5.0 µM/L, whereas valsartan had no effect on the relative phosphorylation of AKT. Relative phosphorylation of ERK was not affected by treatment with either telmisartan or valsartan. To confirm that the inhibition of AKT phosphorylation by telmisartan involves more than AT1 receptor blockade, CHO-K1 cells that lack AT1 receptor were studied. As shown in Figure 6B, telmisartan, but not valsartan, inhibited the relative phosphorylation of AKT. AKT signaling was also compared between smooth muscle cells from SMPGKO mice and control mice to determine whether PPARγ signaling is involved in the activation of AKT. As shown in Figure 6C, telmisartan inhibited phosphorylation of AKT equally in cells from SMPGKO mice and from control mice, reflecting PPARγ-independent effects of telmisartan on AKT activation.

Discussion

In the current study, we investigated the ability of 4 different ARBs to inhibit proliferation of 3 different vascular cell lines of human origin in the absence of supplemental amounts of exogenously added AII. Two of the ARBs tested, telmisartan and irbesartan, are known to have partial agonist activity on PPARγ, whereas the other 2 ARBs tested, eprosartan and valsartan, have little or no effect on PPARγ activity. Telmisartan inhibited vascular cell proliferation in a dose-dependent fashion beginning at concentrations of 2.5 to 5.0 µM/L, and irbesartan showed antiproliferative effects when tested at concentrations >10.0 µM/L. In contrast, eprosartan and valsartan had no effect on the proliferation of human aortic vascular smooth muscle cells even when tested at concentrations ≤80 µM/L, which exceed the levels ordinarily required for robust AT1 receptor blockade. Recently, we reported that telmisartan also dose-dependently inhibited the proliferation of rat vascular smooth muscle cells in the absence of exogenously supplemented AII, whereas ARBs with relatively little or no effect on PPARγ activity did not inhibit cell proliferation. In addition, we found that telmisartan inhibited the proliferation of CHO-K1 cells that
lack AT1 receptors. Taken together, these observations are consistent with the possibility that the antiproliferative effects of telmisartan involve more than just its ability to block AT1 receptors or otherwise interact with AT1 receptors. Other investigators have demonstrated that some ARBs can act as inverse agonists and influence certain AII-independent activities of the AT1 receptor. Given that telmisartan can inhibit proliferation of cells lacking AT1 receptors, it appears that the antiproliferative effects of telmisartan may also go beyond inverse agonism of AT1 receptors. In addition, valsartan, an ARB with known inverse agonist activity, appeared to have relatively little or no ability to inhibit vascular cell proliferation in the absence of AII stimulation.

Given that ARBs with PPARγ agonist activity inhibit proliferation of vascular smooth muscle cell lines in the absence of exogenously supplemented AII, whereas ARBs relatively devoid of PPARγ activity do not, it has been suggested that PPARγ might be playing a role in the antiproliferative effects of molecules like telmisartan. However, because of recent studies showing that telmisartan could inhibit cell proliferation when tested in the presence of a pharmacological inhibitor of PPARγ, the role of PPARγ in the antiproliferative effects of ARBs like telmisartan has been uncertain. In the current studies, we used a genetic approach to directly test whether the antiproliferative effects of telmisartan require the presence of PPARγ. This was accomplished by studying NIH3T3 cells that do not express PPARγ and by studying vascular smooth muscle cells from mice with targeted knockout of PPARγ in smooth muscle. Our findings show that telmisartan, but not valsartan, has the capacity to inhibit proliferation of cells with or without PPARγ.

The fact that telmisartan can inhibit cell proliferation in the absence of the AT1 receptor or in the absence of PPARγ might suggest that the antiproliferative effects of telmisartan observed in these studies do not depend on the presence of either the AT1 receptor or PPARγ. However, an alternative
possibility is that, under the conditions of our studies, the antiproliferative effects of telmisartan require the presence of at least one or the other of these receptors. To investigate this possibility, it will be necessary to test the antiproliferative effects of telmisartan in a double-knockout cell line that lacks both the AT1 receptor and PPARγ, as well as in an otherwise identical control line that expresses one or both of these receptors. In any case, on the basis of previous results and those of the current studies, it now seems clear that the antiproliferative effects of telmisartan involve more than just AT1 receptor blockade and do not require the presence of PPARγ.

In these studies, we further explored potential mechanisms of the antiproliferative effects of telmisartan by investigating its impact on activation of AKT and ERK. 2 key proteins involved in important signaling pathways controlling growth and differentiation. Angiotensin receptor blockade is known to inhibit the ability of supplemental AII to activate AKT and ERK. In addition, Zou et al have shown that, in cardiomyocytes, candesartan can inhibit increases in ERK phosphorylation induced by mechanical stretch in the absence of AII. However, the effects of ARBs on AKT and ERK activation in vascular smooth muscle cells not deliberately stimulated with AII have not been well studied. We have found that telmisartan but not valsartan inhibits activation of AKT but not ERK in vascular smooth muscle cells in the absence of growth stimulation by supplemental amounts of AII. Telmisartan also inhibited activation of AKT in CHO-K1 cells that lack AT1. These findings suggest that the capacity of telmisartan to inhibit AKT activation involves more than just AT1 receptor blockade. We also found that telmisartan can inhibit activation of AKT in smooth muscle cells that lack PPARγ. Akt is a protooncogene with a critical regulatory role in diverse cellular processes, including growth, survival, and the cell cycle. Akt is also a major regulator of insulin signaling and glucose metabolism, and it is activated by phosphorylation at Thr 308 or Ser373, downstream of phosphatidylinositol 3-kinase signaling. Direct targets of telmisartan that result in inhibition of AKT activation remain unknown. Thus, the current findings raise the possibility that inhibition of AKT activity may be a mechanism mediating the antiproliferative effects of telmisartan, including in cells that lack the AT1 receptor or PPARγ, and should motivate future studies on the impact of telmisartan on AT1-related pathways beyond just its ability to block AT1 receptors or activate PPARγ.

Perspectives

It has become increasingly apparent that some ARBs have distinct effects on cellular mechanisms involving more than just AT1 receptor blockade, and that could theoretically contribute to their ability to protect against cardiovascular and metabolic diseases. However, the clinical significance of these cellular effects beyond AT1 receptor blockade remains uncertain, because large-scale, clinical outcome trials based on head-to-head comparisons of different ARBs have never been conducted. In the Ongoing Telmisartan Alone and in Combination With Ramipril Global Endpoint Trial in patients at high risk for cardiovascular disease, clinical outcomes in patients treated with telmisartan were similar to those in patients treated with the angiotensin-converting enzyme inhibitor ramipril. However, angiotensin-converting enzyme inhibitors are also known to influence cardioprotective mechanisms that involve more than just inhibition of the renin-angiotensin system, including effects on bradykinin and NO-related pathways. Thus, the Ongoing Telmisartan Alone and in Combination With Ramipril Global Endpoint Trial could not address whether ARBs like telmisartan provide added clinical benefits beyond those mediated by their ability to inhibit the renin-angiotensin system, because the comparator drug, ramipril, also exerts effects beyond just inhibition of the renin-angiotensin system. Because large numbers of hypertensive patients continue to suffer from adverse cardiovascular events despite treatment with drugs that robustly inhibit the renin-angiotensin system, the availability of next-generation ARBs that protect against cardiovascular disease by doing more than just blocking AT1 receptors could be of significant clinical value. Several next-generation ARBs specifically sought to have added effects beyond just AT1 receptor blockade are currently in development and could hold considerable promise for improving cardiovascular protection in patients with hypertension.

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Telmisartan Induced Inhibition of Vascular Cell Proliferation beyond Angiotensin Receptor Blockade and PPARγ Activation

Koichi Yamamoto*, Mitsuru Ohishi*, Christopher Ho§, Theodore W Kurtz§, Hiromi Rakugi*

From * the department of geriatric medicine, Osaka university graduate school of medicine and § the department of the laboratory medicine, university of California, San Francisco
Supplemental method

Knockout mice
We derived mice with targeted knockout of the gene for PPARγ (Pparg) in smooth muscle cells by crossing transgenic mice expressing Cre recombinase under the control of the mouse transgelin (smooth muscle protein 22-alpha) promoter with mice harboring loxP sites on either side of exons 1 and 2 of Pparg. The transgenic mice expressing Cre recombinase (STOCK Tg(Tagln-cre)1Her/J) and the mice with floxed Pparg (B6.129-Pparg^tm2Rev/J) were purchased from Jackson Laboratories (stock numbers 004746 and 004584, respectively). Hereafter, the STOCK Tg(Tagln-cre)1Her/J mice expressing Cre recombinase under control of the smooth muscle protein 22-alpha (Sm22 alpha) promoter are referred to as Sm22 alpha-Cre mice and the B6.129-Pparg^tm2Rev/J mice with loxP sites flanking Pparg are referred to as floxed Pparg mice. Initially, homozygous Sm22 alpha-Cre mice were bred to homozygous floxed Pparg mice to generate mice with hemizygous Sm22 alpha-Cre and heterozygous floxed Pparg genotypes. These mice were subsequently bred to homozygous floxed Pparg mice to obtain smooth muscle specific Pparg knockout mice (hereafter referred to as SMPGKO mice) and their litter mate controls harboring homogyzous floxed PPARg not expressing Cre recombinase.