ORIGINAL ARTICLE

Telmisartan inhibits cytokine-induced nuclear factor- κ B activation independently of the peroxisome proliferator-activated receptor- γ

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Telmisartan is an angiotensin-II type 1 receptor (AT1R) blocker, currently used to treat patients with hypertension. Telmisartan, in addition to its effect on AT1R, is thought to activate the nuclear transcription factor, peroxisome proliferator-activated receptor- γ (PPAR γ), thereby acting as a partial PPAR γ agonist. This study was conducted to examine whether telmisartan might suppress cytokine-induced inflammatory signaling in vascular endothelial cells, thereby attenuating cellular inflammation possibly by PPAR γ activation. Telmisartan caused a dose-dependent suppression of the tumor necrosis factor- α (TNF α)-induced activation of nuclear factor (NF)- κ B in vascular endothelial cells in this study. The PPAR γ antagonist, GW9662, did not influence the inhibitory effect of telmisartan on NF- κ B activation. The thiazolidinediones neither influenced TNF α -induced NF- κ B activation nor influenced the inhibitory effect of telmisartan in this process. Telmisartan dose dependently diminished the TNF α -induced gene expression of VCAM-1, and GW9662 did not attenuate this effect. Thus, telmisartan inhibits the cytokine-induced expression of the VCAM-1 gene by blocking NF- κ B activation independently of PPAR γ activation. Although the mechanism by which this occurs remains unclear, our findings suggest that telmisartan-induced anti-inflammatory effects might have favorable effects on vasculature in hypertensive patients.

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INTRODUCTION

Atherogenesis results from inflammation within the vessel wall.¹ The renin–angiotensin system (RAS) has an important role in vascular hemostasis. Angiotensin II (Ang II), the main effector of the system, is thought to promote vascular inflammation and atherosclerosis.² Ang II exerts its effects through the Ang type 1 (AT1R) and type 2 (AT2R) receptors.³ The AT1R is the predominant receptor of the cardiovascular system and mediates most of the deleterious effects of Ang II, such as vasoconstriction, endothelial damage and cellular proliferation.³ It has been convincingly shown that inhibition of RAS by an angiotensin-converting enzyme (ACE)-inhibitor or an AT1R antagonist reduces cardiovascular disease.^{4–6}

Telmisartan is an AT1R blocker, currently used to treat patients with hypertension. Recent data suggest that telmisartan, in addition to being an AT1R antagonist, can activate the nuclear transcription factor of peroxisome proliferator-activated receptor- γ (PPAR γ),^{7–9} thereby acting as a partial PPAR γ agonist. PPAR γ is involved in the regulation of glucose homeostasis, in insulin resistance and lipoprotein metabolism. Thus, activators of PPAR γ , such as thiazolidinediones (TZDs), rosiglitazone and pioglitazone, are used clinically in the treatment of diabetes.¹⁰ Furthermore, recent data suggest that telmisartan has anti-inflammatory and antioxidant properties.^{11,12} Therefore, this

study was conducted to examine the effect of telmisartan on cytokine-induced inflammatory signaling, as well as the relationship between cellular inflammation and PPAR γ -activation in vascular endothelial cells.

METHODS

Cell culture

Human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics (San Diego, CA, USA) and cultured in an EGM2 medium supplemented with 2% fetal calf serum (FCS) in the standard manner. The cells in this experiment were used within 3–4 passages and were examined to ensure that they demonstrated the specific characteristics of endothelial cells. SVEC4 cells (murine endothelial cell line; ATCC, Rockville, MD, USA) were also cultured in Dulbecco's modified Eagle's medium containing 10% FCS and were observed to show the typical cobblestone morphological appearance of endothelial cells.¹³

Nuclear factor- κ B activation

To study nuclear factor (NF)- κ B activation, SVEC4 cells were stably transfected with a *cis*-reporter plasmid containing the luciferase reporter gene linked to five repeats of the NF- κ B-binding site (pNF κ B-Luc; Stratagene, La Jolla, CA, USA), as previously described.¹⁴ For this, the pNF κ B-Luc plasmid was transfected,

together with a pSV2neo helper plasmid (Clontech, Palo Alto, CA, USA), into SVEC4 cells using a FuGEN 6 transfection reagent (Boehringer Mannheim, Mannheim, Germany). The cells were then cultured in the presence of G418 (Clontech) at a concentration of 500 mg ml⁻¹ and the medium was replaced every 2–3 days. Approximately 3 weeks after transfection, G418-resistant clones were isolated using a cloning cylinder and analyzed individually for the expression of luciferase activity. Several clones were also selected for the analysis of NF- κ B activation. Luciferase activity was measured using a luciferase assay kit (Stratagene).

We also measured changes in the levels of NF- κ B p50 and p65 in nuclear extracts from HUVECs using a transcription factor assay kit (Active Motif Japan, Tokyo, Japan). Nuclear extracts were prepared with an NE-PER nuclear extraction reagent (Pierce, Rockford, IL, USA), after which p50 and p65 were quantified using a Jurkat nuclear extract as the standard.

Western blot analysis

Human umbilical vein endothelial cells treated with telmisartan for various intervals were lysed using a cell lysis buffer (Cell Signaling, Beverly, MA, USA) with 1 mM phenylmethylsulfonyl fluoride. The protein concentration of each sample was measured using a Bio-Rad detergent-compatible protein assay (Bio-Rad, Tokyo, Japan). Subsequently, β -mercaptoethanol was added to a final concentration of 1%, after which each sample was denatured by boiling for 3 min. Samples containing 10 mg of protein were resolved by electrophoresis on a 12% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad), after which they were incubated with an anti-phospho-Thr-172 AMPK polyclonal antibody (1:1000, Cell Signaling). The binding of each of these antibodies was detected using sheep anti-rabbit IgG in horseradish peroxidase (1:20 000) and an ECL Plus system (Amersham, Buckinghamshire, UK).

Real-time PCR of HUVEC mRNA

For the quantitative measurement of mRNA, 2 μ g of total RNA was treated with DNase I for 15 min and subsequently used for cDNA synthesis. Reverse transcription was carried out using a SuperScript pre-amplification system (Gibco BRL, Gaithersburg, MD, USA) with random oligonucleotide primers. The following primers were used: VCAM-1 forward 5'-GGCAGAGTACG CAAACACTT-3' and reverse 5'-GGCTGTAGCTCCCCGTAG-3', as well as GAPDH forward 5'-GGAGAAGGCTGGGGCTCAT-3' and reverse 5'-TGATGGCATGGACTGTGGTC-3'. A typical reaction (50 μ l) contained 1/50 of reverse transcription (RT)-generated cDNA and 200 nM of primer in a 1 $\times$ SYBR Green RealTime Master Mix (Toyobo, Tokyo, Japan) buffer. The PCRs were carried out in a LineGene system (BioFlux, Tokyo, Japan) under the following conditions: 95°C for 5 min, followed by 40 cycles at 95°C for 15 s, 60°C for 15 s and 72°C for 30 s.

Statistical analysis

Data are presented as mean values $\pm$ s.e.m. Multiple comparisons were evaluated by analysis of variance, followed by Fisher's protected least-significant difference test. A value of $P < .05$ was considered to be statistically significant.

RESULTS

Telmisartan inhibits NF- κ B activation

We initially examined the effects of a 2 h incubation with tumor necrosis factor- α (TNF α) on NF- κ B activation in SVEC4 cells. TNF α induced a sevenfold increase in the NF- κ B-mediated reporter gene expression. Telmisartan dose dependently suppressed the TNF α -induced activation of NF- κ B, although it had no effect on basal NF- κ B activity (Figure 1a).

We also used HUVEC to measure p50 and p65 in nuclear extracts from untreated cells and from cells treated with TNF α in the presence or absence of telmisartan. Marked increases in p50 and p65 were observed 30 min after stimulation with TNF α from very low baseline levels. The observed increases in p50 and p65 were significantly attenuated by telmisartan in a dose-dependent manner (Figure 1b).

Figure 1 (a) Effect of telmisartan on tumor necrosis factor- α (TNF α)-induced nuclear factor (NF)- κ B activation. SVEC4 cells were exposed to various concentrations of telmisartan (0.1–100 μ M) in the presence (closed circles) and absence (open circles) of TNF α . Telmisartan dose dependently suppressed TNF α -mediated NF- κ B-dependent transcriptional activity in SVEC4 cells. (b) HUVECs were stimulated with TNF α in the presence or absence of telmisartan (T30: 30 μ M, T100: 100 μ M) for 30 min. NF- κ B p65 or p50 subunits were then quantified within nuclear extracts using a transcription factor assay kit using the Jurkat nuclear extract as the standard. Data represent the means $\pm$ s.d. ($n=4$). * $P < 0.05$, ** $P < 0.01$.

Effect of a PPAR γ agonist and antagonist on NF- κ B activation

We next examined the effect of the PPAR γ antagonist, GW9662, on the telmisartan-induced inhibition of NF- κ B. GW9662 (10⁻⁶ M) did not influence the inhibitory effect of telmisartan on the NF- κ B-mediated reporter gene expression (Figure 2a).

We also examined the effects of troglitazone, rosiglitazone and pioglitazone on TNF α -induced NF- κ B activation in the presence or absence of telmisartan. The TZDs did not affect TNF α -induced NF- κ B activation and did not attenuate telmisartan-induced NF- κ B activation (Figure 2b).

Effect of duration of incubation with telmisartan and effect of AMP-activated protein kinase

We examined the effect of varying the duration of incubation with telmisartan before and after exposure to TNF α on NF- κ B activity. SVEC4 cells were exposed to telmisartan 180 or 30 min before, at the

Figure 2 (a) Effect of the PPAR γ antagonist, GW9662, on telmisartan-induced nuclear factor (NF)- κ B attenuation. GW9662 (10^{-6} M) did not influence the inhibitory effect of telmisartan on tumor necrosis factor- α (TNF α)-induced NF- κ B activation. White bar: control (no TNF α); black bar: stimulated by TNF α ; hatched bar: stimulated with TNF α in the presence of GW9662. Data represent the means $\pm$ s.d. ($n=4$). * $P<0.05$, ** $P<0.01$ compared with the value obtained in the absence of telmisartan. NS: not significant between the absence and presence of GW9662. (b) The effect of thiazolidinediones on TNF α -induced NF- κ B activation (black bars) and on the telmisartan-mediated inhibition of TNF α -induced NF- κ B activation (white bars). Three PPAR γ agonists (each at a concentration of 10μ M), troglitazone (Tro), rosiglitazone (Rosi) and pioglitazone (Pio), neither influenced TNF α -induced NF- κ B activation nor the inhibitory effect of telmisartan in this process. Data represent the means $\pm$ s.d. ($n=4$). ** $P<0.01$ compared with the value obtained in the absence of telmisartan.

same time or 30 min after the addition of TNF α . A longer pre-incubation with telmisartan caused a more marked suppression of TNF α -induced NF- κ B activation. However, telmisartan still showed a significant inhibitory effect when added 30 min after TNF α (Figure 3a).

Treatment of HUVEC with metformin resulted in a time-dependent activation of AMP-activated protein kinase (AMPK), as monitored by the phosphorylation of AMPK (positive control), whereas telmisartan did not activate AMPK. Accordingly, the AMPK inhibitor compound C did not influence the inhibitory effect of telmisartan on TNF α -induced NF- κ B activation (Figure 3b).

Figure 3 (a) Effect of adding telmisartan (30μ M) at varying intervals before and after exposure to tumor necrosis factor- α (TNF α) on nuclear factor (NF)- κ B activity. Telmisartan was added to SVEC4 cells 180 or 30 min before, at the same time or 30 min after exposure to TNF α . Data represent the means $\pm$ s.d. ($n=4$). (b) Effect of the AMPK inhibitor compound C (CC) on the telmisartan-mediated inhibition of TNF α -induced NF- κ B activation. Inset: Time course analysis for pAMPK by telmisartan or metformin. Data represent the means $\pm$ s.d. ($n=4$). ** $P<0.01$ compared with the control value (TNF α only). NS, not significant between the absence and presence of CC.

Telmisartan inhibits induction of VCAM-1 mRNA

Incubation of HUVEC for 16 h with TNF α substantially induced the gene expression of VCAM-1. However, this TNF α -mediated induction of gene expression was markedly suppressed by a co-treatment with the NF- κ B inhibitor, BAY11-7082, which selectively and irreversibly inhibits cytokine-induced I κ B phosphorylation,¹⁵ suggesting that induction of the VCAM-1 gene is NF- κ B dependent (data not shown). Co-incubation with telmisartan dose dependently diminished the TNF α -induced gene expression of VCAM-1, whereas GW9662 (10^{-6} M) did not influence the inhibitory effect of telmisartan on VCAM-1 gene expression (Figure 4).

DISCUSSION

This study demonstrated the inhibition of TNF α -induced NF- κ B activation by telmisartan in endothelial cells. This anti-inflammatory effect of telmisartan was not mediated by PPAR γ activation, as the

Figure 4 Effect of telmisartan (30 μ M) on tumor necrosis factor- α (TNF α)-induced VCAM-1 mRNA expression in HUVEC. Telmisartan significantly inhibited VCAM-1 mRNA levels. GW9662 (10⁻⁶ M) did not influence the inhibitory effect of telmisartan on TNF α -induced VCAM-1 mRNA expression. White bar: control (no TNF α); black bar: stimulated by TNF α ; hatched bar: stimulated with TNF α in the presence of GW9662. Data represent the means $\pm$ s.e.m. ($n=4$) and are expressed as a ratio of GAPDH. ** $P<0.01$ compared with the value obtained in the absence of telmisartan. NS, not significant between the absence and presence of GW9662.

PPAR γ antagonist, GW9662, had no effect on the telmisartan-mediated inhibition of TNF α -induced NF- κ B activation. The TZDs neither suppressed TNF α -induced NF- κ B activation nor influenced the inhibitory effect of telmisartan in this process. This confirms that telmisartan inhibits TNF α -induced NF- κ B activation independently of PPAR γ activation. Thus, PPAR γ activation probably does not have a large role in the anti-inflammatory effect of telmisartan in endothelial cells, even though TZDs reportedly reduce inflammation in this cell type.^{16,17} Telmisartan has been shown to increase adiponectin levels in humans, perhaps because of an effect on PPAR γ , which shows a high level of expression in adipocytes to produce adiponectin.¹⁸ On the other hand, PPAR γ expression might be limited in endothelial cells, thereby explaining the more important role of an alternative anti-inflammatory mechanism of telmisartan in this cell type.

Angiotensin II by itself induces NF- κ B activation, although to a much lesser extent compared with TNF α . Thus, it is possible for telmisartan to cause an inhibitory signaling on the NF- κ B activation pathway through AT1R. However, this awaits elucidation. Inhibition of the TNF α -induced activation of NF- κ B by telmisartan was directly related to the length of incubation in this study. A longer pre-incubation with telmisartan caused a more marked suppression of TNF α -induced NF- κ B activation. This might suggest that telmisartan induces the production of an anti-inflammatory protein that inhibits NF- κ B activation. However, telmisartan still showed a significant inhibitory effect when added 30 min after exposure to TNF α , suggesting another mechanism of action. We have recently shown that AMPK-activating agents such as metformin,¹⁹ cilostazol²⁰ and fenofibrate²¹ inhibit cytokine-induced NF- κ B activation. Thus, we examined whether telmisartan might also activate AMPK. Telmisartan was not found to activate AMPK as the AMPK inhibitor compound C had no effect on the telmisartan-mediated inhibition of TNF α -induced

NF- κ B activation. It has been recently shown that the interaction between NF- κ B and TNF α signaling induces the activation of IKK β and amplifies oxidative stress, leading to endothelial dysfunction in type 2 diabetics. Thus, telmisartan might inhibit IKK β ; however, it is still to be elucidated.²²

Incubation of HUVEC with TNF α significantly induced VCAM-1 gene expression. The induction of TNF α -induced VCAM-1 mRNA expression is known to be markedly suppressed by co-treatment with the NF- κ B inhibitor, BAY11-7082,¹⁵ suggesting that induction of VCAM-1 mRNA is NF- κ B dependent. Accordingly, telmisartan inhibited VCAM-1 mRNA induction, which was not affected by the PPAR γ antagonist, GW9662.

In conclusion, this study demonstrated that telmisartan inhibits cytokine-induced adhesion molecule gene expression by blocking NF- κ B activation independently of PPAR γ activation in vascular endothelial cells. The mechanism by which this occurs remains unclear. Although experiments on cultured cells do not necessarily reflect what occurs *in vivo*, our findings suggest that telmisartan may have an anti-inflammatory effect on vasculature in hypertensive patients.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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