Telmisartan inhibits the proinflammatory effects of homocysteine on human endothelial cells through activation of the peroxisome proliferator-activated receptor-δ pathway

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Abstract. The aim of this study was to investigate the inhibition capacity of telmisartan to endothelial inflammation induced by homocysteine (Hcy) and discuss the proposed mechanism *in vitro*. Human umbilical vein endothelial cells (HUVECs) were prepared by collagenase digestion and cultured *in vitro*. An increase in monocyte chemoattractant protein-1 (MCP-1) and vascular cell adhesion molecule-1 (VCAM-1) was analyzed by reverse transcription-polymerase chain reaction (RT-PCR) as markers of Hcy-induced endothelial inflammation. HL-60 cell adhesion to HUVECs was measured by rose bengal staining. Nuclear, cytosolic and total nuclear transcription factor-κB (NF-κB) p65 levels were analyzed by western blotting. Peroxisome proliferator-activated receptor-δ (PPARδ) expression by HUVECs exposed to Hcy with or without telmisartan pretreatment was analyzed by RT-PCR and western blotting. Hcy significantly increased the levels of MCP-1 mRNA, VCAM-1 mRNA and monocyte binding to HUVECs. These effects were significantly attenuated by pretreatment with telmisartan and PPARδ agonists. The effect of telmisartan was inhibited by PPARδ antagonists. The Hcy-mediated downregulation of PPARδ mRNA and protein of HUVECs was inhibited by telmisartan. Hcy-mediated upregulation of NF-κB p65 protein levels in nuclear extracts was inhibited by telmisartan and PPARδ agonists. In conclusion, telmisartan exerts potent anti-inflammatory effects in endothelial cells, probably via a binary mechanism involving PPARδ activation and inhibition of the nuclear translocation of NF-κB.

Introduction

Artery atherosclerosis is one of the most common causes of human morbidity and mortality, especially in industrialized societies. Increasing evidence suggests that atherosclerosis is a chronic inflammatory disease of the cardiovascular system and the inflammation related to endothelial cell dysfunction is a key step. Endothelial dysfunction is associated with the induction of adhesion molecules for inflammatory cells, such as intercellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin and fibronectin (1-5).

Peroxisome proliferator-activated receptors (PPARs) are a family of nuclear receptors that have been identified and which function as heterodimers with 9-cis-retinoic receptor (RXR) by binding to a specific PPAR-responsive element in the promoter region of target genes. Three PPAR isoforms have been identified: PPARα, PPARγ and PPARδ. Telmisartan is highly selective for the AII type 1 receptor and it is used for the treatment of hypertension. Recent studies suggest that telmisartan is also a functional PPARγ agonist and experimental evidence has indicated an association with decreased risk for atherosclerosis (6). Additionally, telmisartan influences the expression of PPARγ target genes involved in abdominal adiposity, dyslipidemia and insulin resistance (7,8). A previous study also showed that telmisartan inhibits advanced glycation end-product (AGE)-induced endothelial cell injury by suppressing AGE receptor (RAGE) expression via PPARγ activation without side-effects suhc as thiazolidinedione (the PPARγ agonists) (9), including sodium and water retention. (10,11). These observations indicate that telmisartan functions via an alternative pathway.

As one of the three PPAR isoforms, PPARδ was reported to have non-metabolic functions involved in cell growth, wound healing and inflammation (12,13). Fernández-Fernández (14) found that long-term administration of angiotensin receptor blockers (ARB) significantly decreased visceral fat and

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prevented high-fat diet-induced obesity in wild-type mice and hypertensive rats but not in PPARδ knockout mice. Furthermore, telmisartan, but not losartan or candesartan, increased PPARδ expression in 3T3-L1 preadipocytes. However, no evidence has suggested that telmisartan exerts anti-inflammatory effects via the PPARδ pathway. In this study, we investigated the effects of telmisartan on endothelial inflammation induced by homocysteine stimuli in cultured human umbilical vein endothelial cells (HUVECs) and discussed the proposed mechanism.

Materials and methods

HUVEC isolation and culture. Human umbilical vein endothelial cells (HUVECs) were isolated from full-term human umbilical cords (15-20 cm) obtained immediately post-partum by caesarean section from the first Affiliated Hospital of Fujian Medical University, and stored in M199 medium (Gibco, Gaithersburg, MD, USA). HUVECs were isolated by collagenase digestion of the sub-endothelial basement membrane. Briefly, the umbilical cord vein was cannulated and washed thoroughly using phosphate-buffered saline (PBS; Gibco) to remove blood cells. Sterile M199 medium containing 0.1% collagenase (type I, 10 ml thawed at 37˚C) was infused into the umbilical vein and the other end of the umbilical cord was clamped with a hemostat. The umbilical cord was incubated for 10 min in a 37˚C prior to the cell suspension being collected. Following centrifugation, the cells were resuspended in complete culture medium [M199 with 20% FBS, 0.1 mM L-glutamine, 20 μg/ml endothelial cell growth factor (ECGF), 50 μg/ml sodium heparin]. Cultures were maintained at 37˚C in a humidified CO_2 incubator and 2-4 passages of HUVECs were used in the experiments.

Immunocytochemistry. HUVECs grown in chamber-slides were washed with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature, then washed three times with PBS. Subsequently, the cells were exposed to 0.5% Triton X-100 in PBS for 20 min and washed three times with PBS. The cells were treated with 1% BSA in PBS for 3 min, incubated with specific primary antibody of rabbit anti-VWF (1:200; Dako, Glostrup, Denmark) at 4˚C overnight. After washing, the cells were incubated with goat anti-rabbit IgG-R secondary antibody (1:400; Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA) for 30 min at room temperature. The specimens were observed under a fluorescence microscope (Olympus, Tokyo, Japan) (Fig. 1).

Experimental procedures and reagents. HUVECs, cultured in 6-well tissue culture plates, were preincubated with 1 μM GW0742, 1 μM GSK0660, 1 μM telmisartan or 10 μM PDTC for 30 min and then incubated with 1,000 μM Hcy (all from Sigma) for 24 h.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). A single-step method was used for the isolation of total RNA. Total RNA extraction was achieved using TRIzol reagent according to the manufacturer's instructions (Invitrogen Life Technologies, Grand Island, NY, USA). RNA yield was evaluated spectrophotometrically (A_{260}/A_{280}).

For reverse transcription, 1 μg RNA was incubated with 4 μl PrimeScript buffer, 1 μl PrimeScript RT Enzyme Mix (Takara, Shiga, Japan), 1 μl oligo(dT) primer, and 1 μl random 6-mers. RNase-free dH_2O was then added to produce a final volume of 20 μl. The reaction mixture was incubated at 37˚C for 15 min and then at 85˚C for 5 sec to inactivate the enzyme (15).

Quantitative PCR was performed with 2 μl DNA, 10 μl SYBR Premix Ex Tag™ (Takara), 0.4 μl forward primer, 0.4 μl reverse primer, 0.4 μl ROX Reference Dye. dH_2O was then added to produce a final volume of 20 μl. The reaction was incubated for 30 sec at 95˚C followed by 40 cycles of 5 sec at 95˚C, 30 sec at 60˚C using the following primers for quantitative PCR: MCP-1, 5'-CTTCTGTGCCTGCTGCTCATA-3' and 5'-CTTTGGGACACTTGCTGCTG-3'; VCAM-1, 5'-CGA AAGGCCCAGTTGAAGGA-3' and 5'-GAGCACGAGAAG CTCAGGAGAAA-3'; PPARδ, 5'-AAGGCATCGGGCTTC CACTA-3' and 5'-GCACTTCTGGAAGCGGCAGTA-3'; GAPDH, 5'-GCACCGTCAAGGCTGAGAAC-3' and 5'-TGG TGAAGACGCCAGTGGA-3'. The expression of mRNA levels was measured as the ratio of each mRNA to GAPDH mRNA.

Western blot analysis. Total protein was extracted from HUVECs with ice-cold lysis buffer as previously described (16). For the preparation of nuclear and cytosolic protein extracts, the cells were treated with 200 μl pre-cooled buffer A (containing 1 μl DTT, 10 μl PMSF, 1 μl protease inhibitor per ml) and vortexed for 15 sec. After incubation on ice for 15 min, 11 μl pre-cooled buffer B was added, vortexed for 5 sec and incubated on ice for 1 min. Cytosolic proteins were obtained by centrifuging at 14,000 x g for 5 min at 4˚C. The pellets were resuspended in 100 μl pre-cooled buffer C (containing 1 μl DTT, 10 μl PMSF, 1 μl protease inhibitor per ml) and vortexed for 15 sec. Following incubation at 4˚C for 40 min, the samples were centrifuged at 14,000 x g for 5 min at 4˚C to isolate the supernatant containing nuclear proteins. The samples (20 μl) were loaded and subjected to SDS-PAGE (10% gel). Proteins were electrotransferred to PVDF membranes prior to blocking in TBS-T (5% non-fat dry milk in Tris-buffered saline with Tween-20 for 1 h at room temperature. The membrane was then incubated with primary antibody overnight at 4˚C.

Rabbit anti-nuclear factor-κB (NF-κB) p65 antibody (1:2,000; Cell Signaling Technology, Beverly, MA, USA); rabbit anti-PPARδ antibody (1:200; Abcam, Cambridge, UK); murine anti-β-actin polyclonal antibody (1:1,000) and rabbit anti-histone polyclonal antibody (1:200) (both from Santa Cruz Biotechnology, Inc.). Subsequently, the membrane was washed three times in TBS-T and incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody of goat anti-rabbit IgG or goat anti-mouse IgG (1:2,000; Beijing Zhongshan Biotechnology, Beijing, China). Antigen detection was performed via the ECL reagent and exposed to film (Kodak, Xiamen, China).The band intensities were quantified using a scanning densitometer. β-actin expression was used as an internal standard for total and cytosolic protein, and histone expression was used as an internal standard for nuclear protein.

Adhesion assays. HUVECs were cultured in 96-well tissue culture plates and grown to confluence. The cells were then incubated with GW0742, GSK0660, telmisartan and Hcy for 20 h. HL60 cells ($3x10^5$) were added to each well and incubated
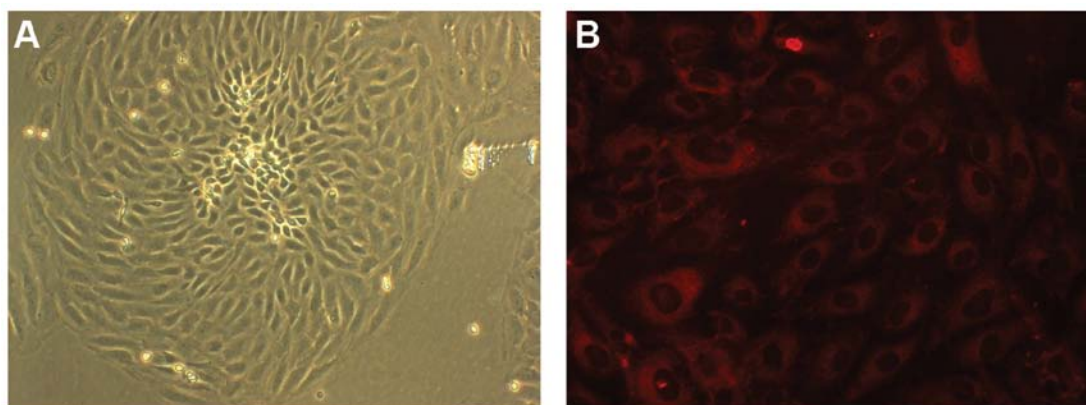
Figure 1. Immunohistochemisty of human umbilical vein endothelial cells (HUVECs). (A) The primary HUVECs were observed under the microscope, and the confluent cells showed presenting a 'pavement stones'. (B) The immunohistochemisty of von Willebrand factor (VWF) were observed under the fluorescence microscope, and red fluorescence was observed in the cytoplasm under the fluorescence microscope.

for 1 h at 37˚C. Non-adherent HL60 cells were removed by washing the wells three times with PBS. Adherence of HL60 cells to HUVECs was photographed under a phase-contrast microscope (Olympus). Rose Bengal (100 μl; 0.25%) stain was added to each well and incubated for 10 min at room temperature. PBS-ethanol (1:1) was added and incubated for 1 h at room temperature after washing the wells. Absorbance (570 nm) was measured using a microplate reader (Bio-Rad, Hercules, CA, USA).

Statistical analysis. Data were expressed as the mean ± SEM. Two-way ANOVA was used for comparisons between the two groups. Data were analyzed using SPSS 18.0 and results were considered statistically significant at $P<0.05$.

Results

Telmisartan attenuates the effect of the increased expression of MCP-1 and VCAM-1 in HUVECs caused by Hcy. Hcy significantly increased the mRNA expression of MCP-1 and VCAM-1 in a concentration-dependent manner (data not shown). Pretreatment with telmisartan significantly attenuated the Hcy-induced MCP-1 and VCAM-1 mRNA expression in a concentration-dependent manner (data not shown).

Effects of telmisartan, PPARδ agonists and antagonists on Hcy-induced MCP-1 and VCAM-1 mRNA expression in HUVECs. The functional significance of the activation of the PPARδ pathway by telmisartan was investigated. Hcy significantly increased the mRNA expression of MCP-1 and VCAM-1. Pretreatment with telmisartan and GW0742 significantly attenuated the Hcy-induced MCP-1 and VCAM-1 mRNA expression, while the effect of telmisartan was not inhibited by GSK0660 (Fig. 2).

Effects of telmisartan, PPAR agonists and antagonists on HUVEC adhesion molecules in vitro. The endothelial inflammation was investigated in Hcy-induced monocyte adhesion assays. HL-60 cell adhesion assays showed significantly increased monocyte binding to confluent HUVECs following
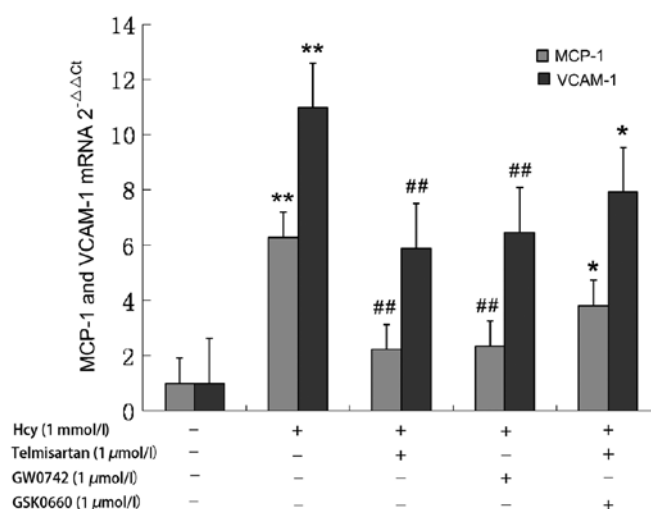

Figure 2. Effects of telmisartan, GW0742 [peroxisome proliferator-activated receptor-δ (PPARδ) agonist] and GSK0660 (PPARδ antagonist) plus telmisartan treatment on homocysteine (Hcy) induced monocyte chemoattractant protein-1 (MCP-1) and vascular cell adhesion molecule-1 (VCAM-1) mRNA expression in human umbilical vein endothelial cells (HUVECs). Incubation with Hcy significantly increased the mRNA expression of MCP-1 and VCAM-1. Telmisartan and GW0742 significantly attenuated the effect of Hcy in HUVECs, while the effect of telmisartan was inhibited by GSK0660 (n=5). **$P<0.01$ vs. control group; ##$P<0.01$ vs. Hcy group; *$P<0.05$ vs. telmisartan group.

Hcy incubation in a concentration-dependent manner (data not shown). Telmisartan and GW0742 significantly reduced the adherence to HUVECs, while the effects of telmisartan were inhibited by GSK0660 (Fig. 3).

Effects of telmisartan and PPAR agonists on PPARδ. RT-PCR and western blot analysis of PPARδ mRNA and protein were performed to investigate the effect of telmisartan on PPARδ. Hcy significantly decreased the mRNA expression of PPARδ, while GW0742 and telmisartan significantly attenuated the effect of Hcy in HUVECs (Fig. 4). The protein expression of PPARδ was also decreased by Hcy, while GW0742 and telmisartan significantly attenuated the effect of Hcy in HUVECs (Fig 5).
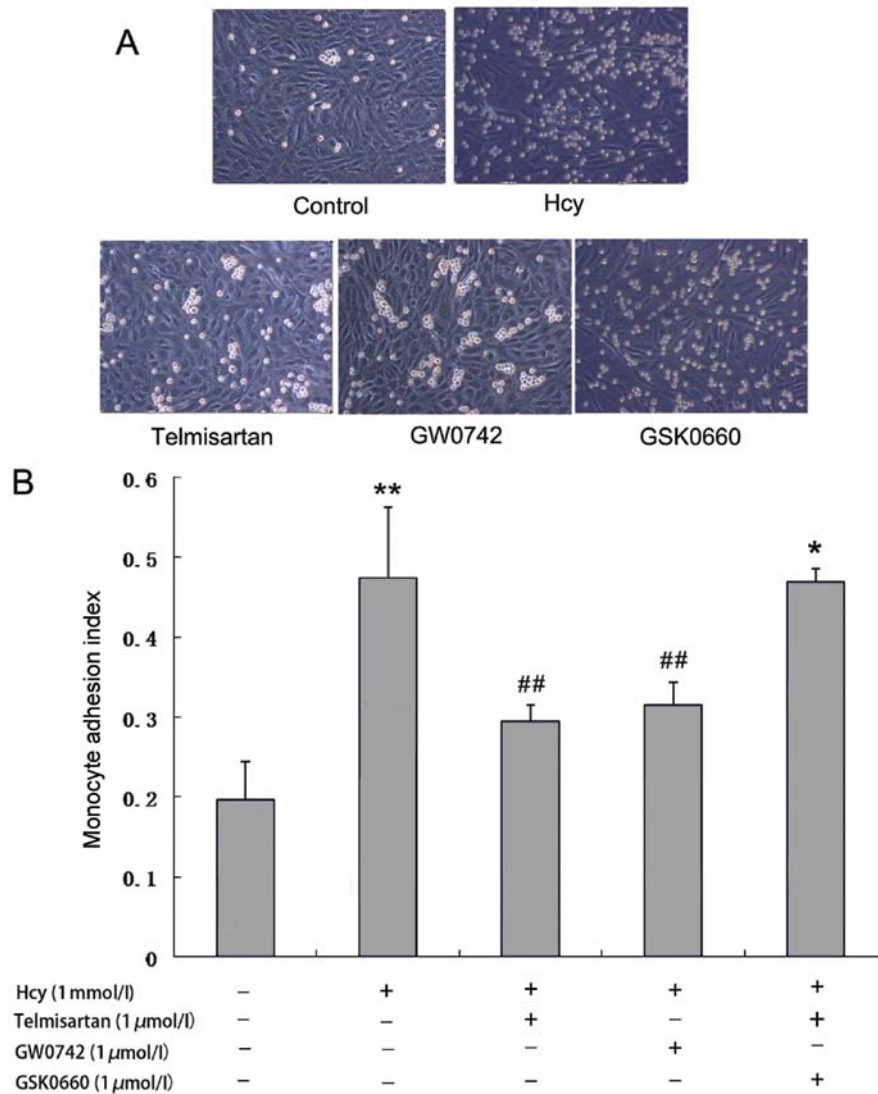
Figure 3. Effects of telmisartan, GW0742 [peroxisome proliferator-activated receptor-δ (PPARδ) agonist] and GSK0660 (PPARδ antagonists) plus telmisartan treatment on homocysteine (Hcy)-induced adhesion molecules expressed by human umbilical vein endothelial cells (HUVECs) *in vitro*. (A) HL-60 cell adhesion assay showed significant increase of monocyte binding to confluent HUVECs after Hcy incubation. (B) Telmisartan and GW0742 significantly reduced the amount of adherence to HUVECs, while the effect of telmisartan was inhibited by GSK0660 (n=3). **P<0.01 vs. control group; ##P<0.01 vs. Hcy group; *P<0.05 vs. telmisartan group.
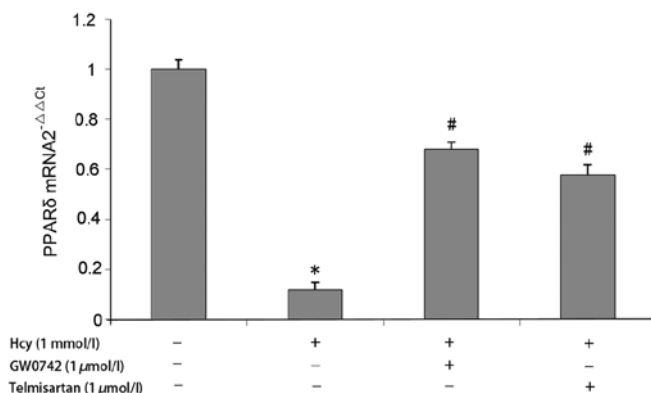

Figure 4. The effect of telmisartan on the expression of peroxisome proliferator-activated receptor-δ (PPARδ) mRNA. RT-PCR was used to analyze the relative gene expression of PPARδ mRNA in human umbilical vein endothelial cells (HUVECs). Incubation with homocysteine (Hcy) significantly decreased PPARδ expression. GW0742 and telmisartan significantly attenuated the effect of Hcy in HUVECs (n=3). *P<0.05 vs. control group; #P<0.05 vs. Hcy group.

Effects of PPARδ agonists and antagonists and NF-κB inhibitors on Hcy-induced mRNA expression of MCP-1 and VCAM-1 in HUVECs. Hcy significantly increased the mRNA expression of MCP-1 and VCAM-1. Pretreatment with GW0742 (PPARδ agonist) and PDTC (NF-κB inhibitor) significantly attenuated the Hcy-induced MCP-1 and VCAM-1 mRNA expression, while GSK0660 (PPARδ antagonist) had no effect (Fig. 6).

Effects of telmisartan, PPARδ agonists and antagonists on Hcy-induced NF-κB p65 translocation in HUVECs. Hcy induced NF-κB p65 protein expression in nuclear proteins. The functional significance of the inhibition of the NF-κB p65 pathway activation by GW0742 was investigated by western blot analysis of NF-κB p65 levels in total, nuclear and cytosolic proteins. Treatment with GW0742 and telmisartan significantly attenuated NF-κB p65 translocation in HUVECs, while GSK0660 had no effect (Fig. 7).
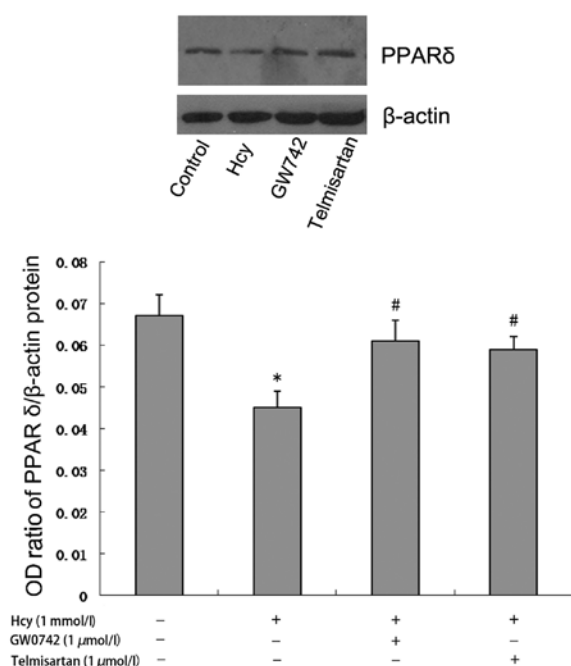
Figure 5. The effect of telmisartan on the expression of peroxisome proliferator-activated receptor-δ (PPARδ) protein. Western blotting was used to measure PPARδ protein and expressed as the ratio of β-actin protein. Incubation with homocysteine (Hcy) significantly decreased PPARδ expression. GW0742 and telmisartan significantly attenuated the effect of Hcy in human umbilical vein endothelial cells (HUVECs) (n=3). $^{*}P<0.05$ vs. control group; $^{#}P<0.05$ vs. Hcy group.

Figure 6. Effects of GW0742 [peroxisome proliferator-activated receptor-δ (PPARδ) agonist], GSK0660 (PPARδ antagonist) and PDTC [nuclear factor-κB (NF-κB) inhibitor] treatment on homocysteine (Hcy)-induced monocyte chemoattractant protein-1 (MCP-1) and vascular cell adhesion molecule-1 (VCAM-1) mRNA expression in human umbilical vein endothelial cells (HUVECs). Incubation with Hcy significantly increased the mRNA expression of MCP-1 and VCAM-1. GW0742 and PDTC significantly attenuated the effect of Hcy in HUVECs, while GSK0660 had no effect (n=5). $^{**}P<0.01$ vs. control group; $^{##}P<0.01$ vs. Hcy group; $^{*}P>0.05$ vs. Hcy group.

Discussion

Telmisartan attenuates the effect of the increased the expression of MCP-1 and VCAM-1 in HUVECs and monocyte adhesion caused by Hcy. Hyperhomocysteinemia (HHcy), which is characterized by abnormally high concentrations of homocysteine in the plasma, is associated with dysfunction of endothelium. The latter causes a chronic inflammatory reaction to cardiovascular system, eventually leading to atherosclerosis of arteries (17,18). In this study, the results show that Hcy significantly increased the expression of MCP-1 and VCAM-1 in HUVECs in a concentration-dependent manner (data not shown). It is well known that an increased expression of chemokines and adhesion molecules is a critical step in inflammatory responses. Moreover, we observed that Hcy significantly increased monocyte binding to confluent HUVECs. Chemokines such as MCP-1 and adhesion molecules such as VCAM-1 play important roles in leukocyte infiltration and activation during the inflammatory processes leading to atherosclerosis (2,3,14). Thus, we hypothesize that Hcy-induced MCP-1 and VCAM-1 expression in endothelial cells may participate in the process of atherosclerosis in patients with hyperhomocysteinemia. Notably, it was observed that telmisartan significantly attenuated Hcy-induced MCP-1 and VCAM-1 expression and reduced cell adherence to HUVECs in a concentration-dependent manner (data not shown). The results were consistent with previous studies that demonstrated that telmisartan pleiotropically modulates tumor necrosis factor-α (TNF-α)-induced VCAM-1 expression and oxidative damage in vascular endothelium by acting
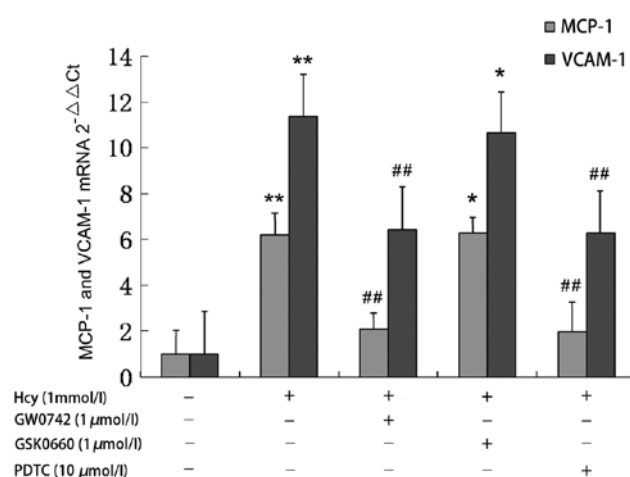
as a hydroxyl radical scavenger (19). However, whether there are other signaling pathways that are involved in the effect of inhibition of inflammation of telmisartan in endothelial cells remains to be clarified.

Telmisartan inhibits the inflammatory activity and effect of monocyte adhesion caused by Hcy via the PPARδ pathway in HUVECs. PPARδ is a family of nuclear receptors that plays a significant role in inflammatory processes and atherosclerosis (20-22). In recent years, understanding of the impact of PPARδ in endothelial dysfunction and atherosclerosis has increased. It was observed that PPARδ mediates resistance to atherosclerosis through upregulation of the 14-3-3 protein ε in human endothelial cells (23). It was also found that telmisartan prevents adipogenesis and weight gain through the activation of PPARδ-dependent signaling pathways (24,25). Thus, we hypothesized that telmisartan is involved in anti-inflammatory effects through the PPARδ pathway. In this study, cells were preincubated with Hcy and PPARδ antagonist GSK0660, followed by incubation with telmisartan. The results showed that the inhibitory effects of telmisartan on the inflammatory activity of Hcy were significantly attenuated by the GSK0660. Investigation of the effects of PPARδ inhibition on the expression of MCP-1 and VCAM-1 induced by Hcy in vascular endothelial cells revealed no significant differences compared to the Hcy-treated group (Fig. 2). On the other hand, Fan *et al* (26) revealed that the PPARδ agonists GW0742 suppressed TNF-α-induced ensuing leukocyte recruitment. Similarly, results of this study have shown that PPARδ agonists GW0742 or telmisartan significantly attenuated monocyte adhesion induced by Hcy, while the effect was significantly inhibited by GSK0660 (Fig. 3). The results indicate that telmisartan is important in inhibition of inflammation via PPARδ pathway. To confirm the PPARδ activation
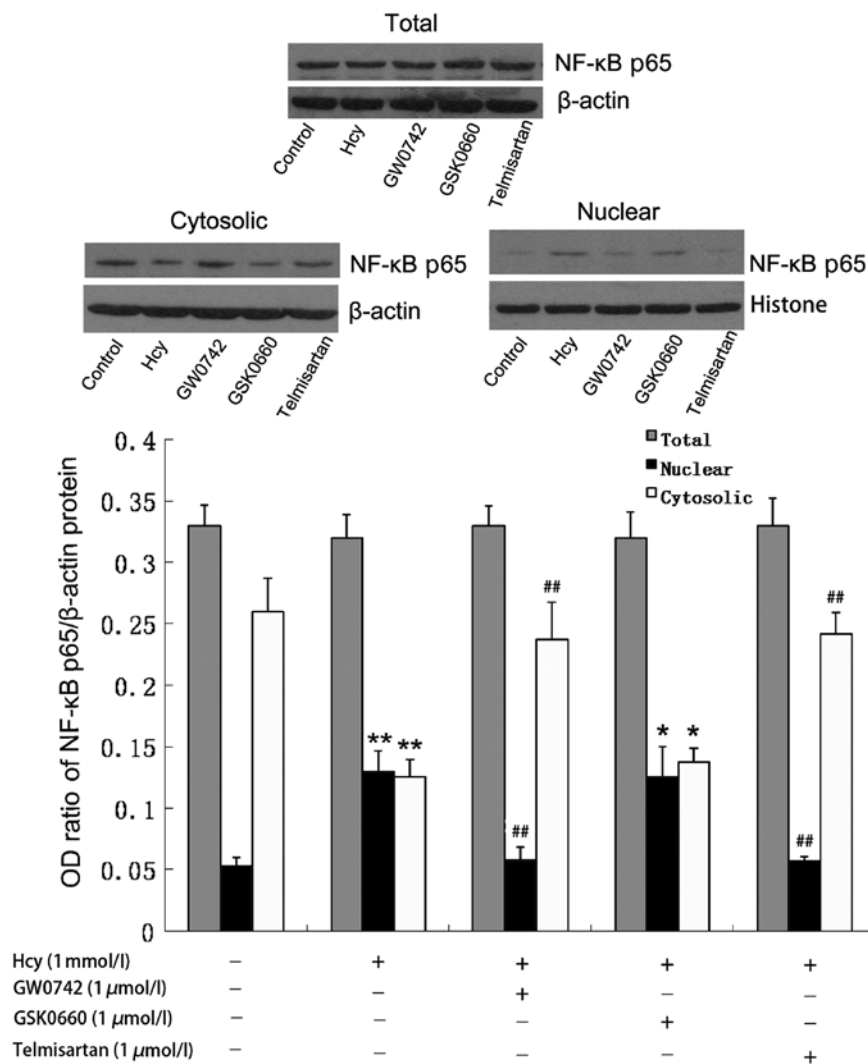
Figure 7. Effects of GW0742 [peroxisome proliferator-activated receptor-δ (PPARδ) agonist], GSK0660 (PPARδ antagonist) and telmisartan on homocysteine (Hcy)-induced nuclear factor-κB (NF-κB) p65 translocation in human umbilical vein endothelial cells (HUVECs). Expression of NF-κB p65 protein levels were measured as the ratio of β-actin or histone protein. Hcy increased the amount of NF-κB p65 in nuclear protein. Treatment with GW0742 and telmisartan significantly attenuated NF-κB p65 translocation in HUVECs, whereas GSK0660 had no effect (n=3). **P<0.01 vs. control group; $^{##}$P<0.01 vs. Hcy group; *P>0.05 vs. Hcy group.

effect of telmisartan, we compared the impact of telmisartan and PPAR agonists on the expression of PPARδ mRNA and protein. It was demonstrated that incubation with Hcy significantly decreased PPARδ expression in vascular endothelium, and the effect was inhibited by telmisartan and GW0742 (Figs. 4 and 5). The results strongly suggest that telmisartan inhibits the inflammatory activity of Hcy partly via the PPARδ pathway in vascular endothelium.

Activation of PPARδ caused by telmisartan may interfere with inflammatory gene transcription via the inhibition of nuclear translocation and activation of NF-κB. To investigate the role of PPARδ in the proinflammatory effects of vascular endothelium cells induced by Hcy, the correlation between PPARδ and NF-κB was investigated. In quiescent endothelial cells, NF-κB resides in the cytoplasm in an inactive form that is associated with an inhibitory protein (IκB). Following stimulation, IκB is phosphorylated and degraded by the proteasome, thus allowing translocation of the dissociated NF-κB to the nucleus in an active form resulting in regulation of the expression of target genes including cytokines, adhesion molecules and chemokines (27). In this study, pretreatment with GW0742 and NF-κB inhibitor PDTC significantly attenuated the Hcy-induced MCP-1 and VCAM-1 mRNA expression, suggesting the effect of PPARδ and NF-κB in the process of inhibition of inflammation (Fig. 6). Determination of NF-κB p65 levels in total, nuclear and cytosolic proteins revealed that the activation of PPARδ inhibits Hcy-induced nuclear translocation of NF-κB in addition to inhibiting the inflammatory action of Hcy (Fig. 7). These data suggest that PPARδ interferes with the transcription of inflammatory genes by inhibiting nuclear translocation and activation of NF-κB and NF-κB may be a downstream signaling molecule of PPARδ in the pathway of inflammation inhibition. The above results are similar to those of a previous study, which reported that the PPARδ agonist GW501516 inhibited the TNF-α-mediated upregulation of mRNA levels of the NF-κB target gene IL-8 via decreased p65 acetylation in cultured human HaCaT keratinocytes *in vitro* (28).

In conclusion, the results of this study have demonstrated that telmisartan suppresses the Hcy-induced expression of the

proinflammatory adhesion molecules MCP-1 and VCAM-1 in endothelial cells and attenuates the effect of monocyte adhesion. The underlying mechanism is a binary process involving the activation of PPARδ and the prevention of the nuclear translocation of NF-κB.

Acknowledgements

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