Inhibition of Tumor Necrosis Factor-α–Induced Interleukin-6 Expression by Telmisartan Through Cross-Talk of Peroxisome Proliferator-Activated Receptor-γ With Nuclear Factor κB and CCAAT/Enhancer-Binding Protein-β

Qingping Tian, Ryohei Miyazaki, Toshihiro Ichiki, Ikuyo Imayama, Keita Inanaga, Hideki Ohtsubo, Kotaro Yano, Kotaro Takeda, Kenji Sunagawa

Abstract—Telmisartan, an angiotensin II type 1 receptor antagonist, was reported to be a partial agonist of peroxisome proliferator-activated receptor-γ. Although peroxisome proliferator-activated receptor-γ activators have been shown to have an anti-inflammatory effect, such as inhibition of cytokine production, it has not been determined whether telmisartan has such effects. We examined whether telmisartan inhibits expression of interleukin-6 (IL-6), a proinflammatory cytokine, in vascular smooth muscle cells. Telmisartan, but not valsartan, attenuated IL-6 mRNA expression induced by tumor necrosis factor-α (TNF-α). Telmisartan decreased TNF-α–induced IL-6 mRNA and protein expression in a dose-dependent manner. Because suppression of IL-6 mRNA expression was prevented by pretreatment with GW9662, a specific peroxisome proliferator-activated receptor-γ antagonist, peroxisome proliferator-activated receptor-γ may be involved in the process. Telmisartan suppressed IL-6 gene promoter activity induced by TNF-α. Deletion analysis suggested that the DNA segment between −150 bp and −27 bp of the IL-6 gene promoter that contains nuclear factor κB and CCAAT/enhancer-binding protein-β sites was responsible for telmisartan suppression. Telmisartan attenuated TNF-α–induced nuclear factor κB– and CCAAT/enhancer-binding protein-β–dependent gene transcription and DNA binding. Telmisartan also attenuated serum IL-6 level in TNF-α–infused mice and IL-6 production from rat aorta stimulated with TNF-α ex vivo. These data suggest that telmisartan may attenuate inflammatory process induced by TNF-α in addition to the blockade of angiotensin II type 1 receptor. Because both TNF-α and angiotensin II play important roles in atherogenesis through enhancement of vascular inflammation, telmisartan may be beneficial for treatment of not only hypertension but also vascular inflammatory change. (Hypertension. 2009;53: 00-00.)

Key Words: interleukin-6 ■ TNF-α ■ PPARγ ■ NF-κB ■ C/EBPβ

Angiotensin II (Ang II) is a main final effector molecule of the renin–angiotensin system. Physiologically, Ang II plays an important role in the regulation of blood pressure, fluid volume, and electrolyte balance. However, Ang II is also involved in the pathological processes, such as cardiovascular diseases, renal insufficiency, and metabolic disorders. Indeed, inhibition of the renin–angiotensin system by Ang II type 1 receptor (AT1R) antagonists has been proven beneficial for treatment of heart failure, chronic kidney diseases, and myocardial infarction. AT1R antagonists also showed favorable effects on prevention of new onset of diabetes mellitus and atrial fibrillation.

Telmisartan, one of the AT1R antagonists, was reported to be a partial agonist of peroxisome proliferator-activated receptor-γ (PPARγ). PPARγ is a nuclear receptor transcription factor, and the target genes of PPARγ are involved in the regulation of lipid and glucose metabolism and adipocyte differentiation. In addition, it is reported that thiazolidinediones (TZDs), synthetic PPARγ ligands, have an anti-inflammatory effect and inhibit atherogenesis. The anti-inflammatory effect of TZDs involves inhibition of the function of nuclear factor κB (NF-κB), which plays an important role in the expression of many genes mediating an inflammatory process.

Interleukin-6 (IL-6) is one of the proinflammatory cytokines and is induced by tumor necrosis factor-α (TNF-α), Ang II, and other stimuli in vascular smooth muscle cells (VSMCs), endothelial cells, and macrophages. IL-6 plays an...
important role in vascular remodeling and was reported to be a useful biomarker in predicting future cardiovascular events.15

Telmisartan has been shown to induce differentiation of adipocytes through activation of PPARγ. A recent study showed that telmisartan attenuated hepatic steatosis, inflammation, and fibrosis in a rat model of nonalcoholic steatohepatitis.16 It was also reported that telmisartan treatment of patients with hypertension and coronary heart disease decreased β2-integrin MAC-1 expression in peripheral lymphocytes independent of Ang II.17 These data suggest that telmisartan has an anti-inflammatory effect independently of AT1R blocking effect. However, an anti-inflammatory effect of telmisartan on blood vessel is incompletely characterized. Therefore, we tested whether telmisartan inhibits TNF-α–induced IL-6 expression through PPARγ in VSMCs.

Materials and Methods
DMEM was purchased from Gibco/BRL. FBS was from JRH Biosciences. Recombinant TNF-α was a generous gift from Dainippon-Sumitomo Pharmaceutical Co (Osaka, Japan). Telmisartan was a generous gift from Boehringer Ingelheim (Ingelheim, Germany). Valsartan was purchased from US Pharmacopeia. BSA and GW9662 were purchased from Sigma. Pioglitazone was purchased from LKT Laboratories. [32P]dCTP and [32P]ATP were purchased from Perkin-Elmer Life Sciences. Antibodies against extracellular signal-regulated protein kinase (ERK), p38 mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), and their phosphorylated forms were purchased from Cell Signaling Technology. Other reagents were purchased from Wako Pure Chemicals unless otherwise mentioned specifically. TNF-α was dissolved in dimethyl sulfoxide at a final concentration of 0.1%, which did not show any effect on IL-6 induction.

Cell Culture
VSMCs were isolated from the thoracic aorta of Sprague-Dawley rats and cultured in a humidified atmosphere of 95% air/5% CO2 at 37°C in DMEM as described previously.14 Cells were grown to confluence and cultured in a humidified atmosphere of 95% air/5% CO2 at 37°C in DMEM supplemented with 0.1% BSA for 2 days before use. Other reagents that added to culture medium were dissolved in dimethyl sulfoxide at a final concentration of 0.1%, which did not show any effect on IL-6 induction.

Northern Blotting
Total RNA was prepared according to the acid guanidinium thiocyanate-phenol-chloroform extraction method. Northern blot analysis of IL-6 mRNA and 18S ribosomal RNA (rRNA) was performed as described previously.14 The radioactivity of hybridized bands of IL-6 mRNA and rRNA was quantified with a MacBAS Bioimage Analyzer (Fuji Photo Film). It was reported that 2 species of IL-6 mRNA were generated by an alternative polyadenylation.18 The intensity of both bands was taken into account for quantification.

Quantification of Rat IL-6 by Sandwich ELISA
VSMCs were stimulated with TNF-α (10 ng/mL) or Ang II (100 nmol/L) for 24 hours in the presence or absence of telmisartan (1 to 20 μmol/L). Then the medium of VSMCs was collected and centrifuged at 12 000 rpm for 1 minute. The supernatant was stored at −70°C until used for the assay. ELISA for rat IL-6 was performed with a Cytoscreen ELISA kit (BioSource International) according to manufacturer instructions. The measurement was performed in duplicate.

Transfection of IL-6 Promoter–Luciferase Fusion DNA Construct to VSMCs
The IL-6 gene promoter–luciferase fusion DNA constructs and luciferase assay were described previously.14 Detailed protocols can be found in an online data supplement available at http://hyper.ahajournals.org.

Plasmids of NF-kB–luciferase and CCAAT/enhancer-binding protein-β (C/EBPβ)–luciferase were purchased from Stratagene Co. Five copies of NF-kB consensus sequence or 3 copies of C/EBPβ consensus sequence were ligated to minimal promoter followed by luciferase gene.

Gel Mobility Shift Assay
Gel mobility shift assay was performed as described previously14 using synthetic NF-kB and C/EBPβ DNA probe (NF-kB: CAT GTG GGA TTT TCC CAT GA; C/EBPβ: CAC ATT GCA CAA TCT TAA). Detailed protocols are indicated in the online supplement.

Effect of Telmisartan on Ang II– and TNF-α–Induced IL-6 Production In Vivo
All procedures were approved by the institutional animal use and care committee and were conducted in conformity with institutional guidelines of Kyushu University. Ang II (490 ng/kg per minute) or TNF-α (80 ng/kg per minute) was administered subcutaneously to 9-week-old C57BL/6J mice (Kyudo Co; Saga, Japan) by osmotic mini-pump (Alzet) for 1 week. Doses of TNF-α and Ang II were determined in a preliminary experiment to detect a significant increase in the serum IL-6 level. Telmisartan was dissolved in water (10 μg/mL) and administered ad libitum. The estimated dose of orally ingested telmisartan was 2 mg/kg per day. Blood pressure and heart rate were measured using tail-cuff method (UR-5000; UEDA). After 1 week, mice were euthanized under pentobarbital anesthesia, and peripheral blood was collected from inferior vena cava. The serum concentration of IL-6 was measured using ELISA kit (R&D Systems). No significant differences in body weight were observed among the treatment groups (data not shown).

Ex Vivo Stimulation of Rat Aorta
Nine-week-old Sprague-Dawley rats were purchased from Kyudo Co. Rats were euthanized under deep pentobarbital anesthesia. The aorta was excised and adventitia was removed. The aorta was cut into 6 pieces and stimulated with TNF-α (50 ng/mL) or Ang II (1 μmol/L) in the absence or presence of telmisartan (10 μmol/L) in 500 μL of DMEM supplemented with 0.1% BSA for 48 hours. Concentrations of Ang II and TNF-α were determined in a preliminary experiment to detect a significant increase in the production of IL-6 in the supernatant of ex vivo–cultured aortic segments. The supernatant was subjected to ELISA to measure IL-6 production. The IL-6 concentration in the supernatant was normalized with the wet weight of the aortic segment.

RT-PCR and Western Blot Analysis
Detailed protocols are indicated in the online supplement.

Statistical Analysis
Statistical analysis was performed with 1-way ANOVA and Fisher’s test if appropriate. A P value <0.05 was considered statistically significant. Values are expressed as mean±SEM.

Results
Telmisartan Attenuated TNF-α–Induced IL-6 Expression
VSMCs were incubated with or without telmisartan (10 μmol/L) for 60 minutes. Then the cells were stimulated with TNF-α (10 ng/mL) for 30 minutes. Northern blot analysis revealed attenuation of TNF-α–induced IL-6 mRNA expression by telmisartan (Figure 1A). However, valsartan (10 μmol/L), another AT1R antagonist, failed to suppress TNF-α–induced IL-6 mRNA expression (Figure 1B). Telmisartan (1 to 20 μmol/L) dose-dependently suppressed TNF-α–induced IL-6 mRNA expression (Figure 1C). The concentration range of telmisartan was chosen based on a previous
clinical study\textsuperscript{19} that showed that the steady-state serum level of telmisartan was 1 to 5 µmol/L when 80 to 160 mg per day of telmisartan was given for 7 days to patients with essential hypertension. And it was reported that telmisartan at concentrations >25 µmol/L stimulated PPAR\textgamma.\textsuperscript{8} Therefore, we did not use telmisartan at concentrations >20 µmol/L in this study.

The protein level of IL-6 in the supernatant of VSMCs was measured after 24 hours of stimulation with TNF-\textalpha (10 ng/mL) with or without preincubation with telmisartan (1 to 20 µmol/L). TNF-\textalpha-induced IL-6 protein expression was also dose-dependently attenuated by telmisartan (Figure 2A). Ang II (100 nmol/L)-induced IL-6 production was inhibited completely by telmisartan at concentrations >1000 ng/mL in this study.

Next, we examined whether telmisartan affected TNF receptor expression. Semiquantitative RT-PCR analysis showed that telmisartan did not affect TNF type 1 receptor mRNA expression (supplemental Figure 1B). We could not detect TNF type 2 receptor mRNA in our VSMCs. We also examined the effect of telmisartan on TNF-\textalpha-induced MAPK activation (supplemental Figure II). Telmisartan did not affect TNF-\textalpha-induced activation of ERK, p38MAPK, or JNK.

**Figure 1.** Suppression of TNF-\textalpha-induced IL-6 mRNA expression by telmisartan (Tel). VSMCs were preincubated with Tel (10 µmol/L; A), valsartan (Val; 10 µmol/L; B), or various concentrations (1 to 20 µmol/L; C) of telmisartan for 60 minutes and stimulated with TNF-\textalpha (10 ng/mL) for 30 minutes. Total RNA was isolated, and expression of IL-6 mRNA and 18S rRNA was determined by Northern blot analysis. Radioactivity of IL-6 mRNA was measured with an imaging analyzer and was normalized by radioactivity of rRNA. Values (mean±SEM) are expressed as percentage of control culture in a bar graph (100%; No. of independent experiments was 5). \(*P<0.05; **P<0.01\) vs control; \#P<0.05; ##P<0.01 vs TNF-\textalpha.

**Figure 2.** Suppression of TNF-\textalpha and Ang II-induced IL-6 protein production by telmisartan (Tel). A, VSMCs were preincubated with Tel (10 µmol/L) at various concentrations for 60 minutes and stimulated with TNF-\textalpha (10 ng/mL) for 24 hours. B, VSMCs were incubated with Tel at 1 or 5 µmol/L and stimulated with Ang II (100 nmol/L) for 24 hours. IL-6 protein production in the supernatant of VSMCs was measured by ELISA. \(*P<0.05\) vs control; \(**P<0.01\) vs control; \#P<0.05 vs TNF-\textalpha; ##P<0.01 vs TNF-\textalpha or Ang II (No. of independent experiment was 8 in duplicate).

**Telmisartan Inhibition of TNF-\textalpha-Induced IL-6 Expression Was Dependent on PPAR\textgamma**

To clarify the role of PPAR\textgamma in telmisartan inhibition of TNF-\textalpha-induced IL-6 expression, the effect of GW9662, a PPAR\textgamma-specific antagonist, was examined. Although GW9662 itself did not affect IL-6 mRNA expression, preincubation with GW9662 (10 µmol/L; 3 hours) blocked telmisartan inhibition of TNF-\textalpha-induced IL-6 expression (Figure 3A). Pioglitazone (10 µmol/L; preincubation for 1 hour), a full PPAR\textgamma agonist, also suppressed the TNF-\textalpha-induced IL-6 mRNA expression (Figure 3B).

**Telmisartan-Inhibited IL-6 Gene Promoter Activity**

Next, the effect of telmisartan on IL-6 gene promoter activity was examined. TNF-\textalpha (10 ng/mL) increased IL-6 gene promoter activity by 2-fold. Preincubation with telmisartan (10 µmol/L) significantly inhibited IL-6 gene promoter activity (Figure 4). Deletion analysis of the IL-6 gene promoter suggested that the DNA segment between −150 bp and −27 bp was responsible for the downregulation by telmisartan (Figure 4A) because telmisartan inhibited the luciferase activity in the −150-bp construct, but the −27-bp...
construct no longer responded to TNF-α or telmisartan. The DNA segment between −150 bp and −27 bp contains NF-κB and C/EBPβ as consensus cis DNA elements. We therefore examined whether telmisartan inhibited NF-κB– and C/EBPβ–dependent gene transcription activated by TNF-α. As shown in Figure 4B, telmisartan inhibited TNF-α–induced activation of luciferase activity, which is solely dependent on NF-κB or C/EBPβ.

The gel mobility shift assay showed that telmisartan inhibited TNF-α–induced NF-κB DNA binding activity (Figure 5A). Telmisartan also attenuated TNF-α–induced C/EBPβ DNA binding activity to a lesser extent (Figure 5B).

Telmisartan Attenuated IL-6 Production In Vivo and Ex Vivo
To confirm that telmisartan inhibits IL-6 production in vivo, Ang II (490 ng/kg per minute) or TNF-α (80 ng/kg per minute) was administered to mice with or without telmisartan (2 mg/kg per day) for 1 week. Ang II but not TNF-α increased blood pressure level (Table). Ang II–induced high blood pressure was inhibited by telmisartan. Heart rate was not significantly different among the treatment groups. Ang II–induced increase in serum IL-6 level was almost completely inhibited by telmisartan, and telmisartan significantly attenuated TNF-α–induced IL-6 production (Figure 6A).
confirm that IL-6 is produced from blood vessel, a segment of rat aorta without adventitia was stimulated ex vivo with Ang II (1 μmol/L) or TNF-α (50 ng/mL) in the presence or absence of telmisartan (10 μmol/L) for 48 hours. Production of IL-6 induced by TNF-α in the supernatant was significantly attenuated by coincubation with telmisartan (Figure 6B). Ang II–induced production of IL-6 was completely inhibited by telmisartan. These results were consistent with those obtained during in vitro experiments.

Discussion

In the present study, we demonstrated that telmisartan but not valsartan suppressed TNF-α-induced IL-6 expression through a PPARγ-dependent manner. Inhibition of NF-κB and C/EBPβ DNA binding activity by telmisartan may be responsible for attenuation of TNF-α–induced IL-6 expression. This is the first study demonstrating that telmisartan modulates cytokine production induced by non–Ang II stimuli. The in vivo and ex vivo results were consistent with those obtained from the in vitro study. The in vivo study showed that telmisartan had an anti-inflammatory effect in mice, and the ex vivo study indicated that IL-6 was produced from blood vessel in response to TNF-α stimulation, and telmisartan attenuated the induction.

On activation by ligands, PPARγ regulates expression of several genes involved in lipid and carbohydrate metabolism and inflammatory responses.21 PPARγ regulates gene expression through 2 different transcriptional regulatory mechanisms: transactivation and transrepression. Transactivation depends on PPARγ response element. On activation, PPARγ forms a heterodimer with retinoid X receptor and binds to PPARγ response element in the promoter region of the target genes.22 In contrast, transrepression involves an interference with other transcription factors such as NF-κB and activator protein 1.23 Although telmisartan was reported to be a partial agonist of PPARγ, it has not been determined whether telmisartan regulates gene expression through transrepression mechanism. Our data suggest that telmisartan may have a transrepression effect on gene expression in addition to AT1R blockade.

The mechanism of transrepression by PPARγ activators is less well known. A recent study showed that PPARγ activation by TZD induced sumoylation of PPAR, resulting in retention of nuclear receptor corepressor/histone deacetylase complex to the promoter and suppression of gene transcription.24 Troglitazone, another TZD, inhibited TNF-α–induced and NF-κB–dependent gene transcription without affecting NF-κB nuclear translocation or DNA binding in adipocytes,23 which may support the above-mentioned model. However, a previous study showed that TZDs inhibited IL-1β–activated NF-κB and C/EBPβ DNA binding to the IL-6 gene promoter.24 It was also reported that troglitazone inhibited TNF-α–induced IL-6 expression in multiple myeloma cells by inhibiting NF-κB and C/EBPβ DNA binding.25 In this study, activated PPARγ competed for PPARγ coactivator-1, a transcription coactivator, with NF-κB, resulting in attenua-

| Table. Heart Rate and Blood Pressure of Ang II– and TNF-α–Treated Mice |
|-----------------------------|---------|---------|-----------|-----------|---------|---------|
| Variable        | Control | Tel     | Ang II   | Tel + Ang II | TNF-α   | Tel + TNF-α |
| HR (bpm)        | 576±24  | 598±21  | 599±18   | 608±27    | 611±47  | 586±22   |
| BP (mm Hg)      | 95.3±1.3| 94.0±2.8| 109.6±4.7*| 102.2±1.8†| 97.3±0.9| 95.5±1.4 |

HR indicates heart rate; BP, blood pressure.
*P<0.05 vs control; †P<0.05 vs Ang II; n=5.
activated PPAR\gamma, suggesting that this protein–protein interaction attenuates the inflammatory and proliferative responses. IL-6 enhanced VSMC proliferation and DNA synthesis of VSMCs, which may coordinate increased both monocyte chemoattractant protein-1 production by TNF-α–induced NF-κB DNA binding. In contrast, activated PPAR\gamma physically interacted with C/EBPβ, suggesting that this protein–protein interaction attenuates the DNA binding of C/EBPβ. Although the precise mechanisms are not clear at this point, it may be possible that telmisartan inhibits NF-κB and C/EBPβ DNA binding activity through the same mechanism.

Inflammation plays a crucial role in the initiation and progression of atherosclerosis. IL-6 enhances VSMC growth induced by platelet-derived growth factor. IL-6 also increased both monocyte chemoattractant protein-1 production and DNA synthesis of VSMCs, which may coordinate inflammatory and proliferative responses. IL-6 is also a useful biomarker predicting future cardiovascular events. TNF-α also enhances vascular inflammation. Blockade of TNF-α activity by soluble TNF-α receptor suppressed coronary artery neointimal formation after cardiac transplantation in rabbits. Therefore, telmisartan inhibition of TNF-α–induced IL-6 expression, which was not observed by valsartan, may attenuate vascular inflammation.

A recent report showed that C/EBPβ was involved in IL-17–induced C-reactive protein expression in VSMCs. Another report showed that C/EBPβ regulated monocyte chemotactic protein-1 expression in the aorta of hyperinsulinemic rats. These studies suggest that C/EBPβ is also involved in vascular inflammation. Because NF-κB is well known to regulate gene expression of various inflammatory molecules, telmisartan inhibition of NF-κB and C/EBPβ may contribute to attenuation of a broad range of inflammatory responses of blood vessel. However, it is not clear at this point whether telmisartan modulates gene expression induced by TNF-α other than IL-6 induction.

TZDs were constantly reported to inhibit atherogenesis in various models. Rosiglitazone inhibited development of atherosclerosis in LDL receptor–deficient mice. Rosiglitazone was also shown to have additive effects on plaque regression in the combination treatment with simvastatin in an atherosclerotic rabbit model. AT1R antagonists were also reported to suppress atherogenesis. Strawn et al demonstrated that losartan attenuated atherogenesis in monkeys with hypercholesterolemia. Based on these studies and our results, telmisartan may be more protective against vascular lesion formation attributable to PPAR\gamma activation and AT1R antagonism.

**Perspective**

In the present study, we showed that telmisartan inhibited Ang II– as well as TNF-α–induced IL-6 expression in VSMCs, rat aorta, and mice. Inhibition of TNF-α–induced IL-6 expression was mediated by PPAR\gamma. And inhibition of NF-κB and C/EBPβ DNA binding by telmisartan may be responsible for suppression of TNF-α–induced IL-6 expression. The dual inhibition (Ang II– and TNF-α–induced IL-6 expression) of the inflammatory cytokine production by telmisartan may be beneficial for treatment of not only hypertension but also atherosclerotic cardiovascular diseases. However, large clinical trials are needed to determine whether these unique properties of telmisartan cause better clinical outcome in cardiovascular disease prevention.

**Sources of Funding**

This study was supported in part by grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (19590867; T.I.). Q.T. was supported by the Japan–China Sasakawa Medical Fellowship.

**Disclosures**

None.

**References**


**Figure 6.** Telmisartan attenuated IL-6 production in vivo and ex vivo. α. Serum concentration of IL-6 was measured in mice injected with Ang II (490 ng/kg per minute) or TNF-α (80 ng/kg per minute) in the presence or absence of telmisartan (Tel; 2 mg/kg per day) administration for 1 week (No. of independent experiments was 5). *P<0.05 vs Ang II; **P<0.01 vs control (no treatment); #P<0.05 vs Ang II; ††P<0.01 vs TNF-α. UD indicates undetectable. B, An aortic segment was stimulated ex vivo with Ang II (1 µmol/L) or TNF-α (50 ng/mL) in the presence or absence of Tel (10 µmol/L) in DMEM supplemented with 0.1% BSA for 48 hours. The concentration of IL-6 in the supernatant was measured and normalized with wet weight of the aortic segment (No. of independent experiment was 4 in duplicate). **P<0.01 vs control; #P<0.05 vs Ang II; †P<0.05 vs TNF-α.


Inhibition of Tumor Necrosis Factor-α–Induced Interleukin-6 Expression by Telmisartan Through Cross-Talk of Peroxisome Proliferator-Activated Receptor-γ With Nuclear Factor κB and CCAAT/Enhancer-Binding Protein-β

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Hypertension. published online March 16, 2009:
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/early/2009/03/16/HYPERTENSIONAHA.108.126656.citation

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Supplemental File For

Inhibition of TNF α-Induced Interleukin-6 Expression by Telmisartan through Crosstalk of PPARγ with NF-κB and C/EBPβ

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Supplemental Methods

Transfection of IL-6 Promoter-Luciferase Fusion DNA Construct to VSMCs
VSMCs (5x10^5) were prepared in a 6 cm-tissue culture dish. After 48 hours, IL-6 promoter-luciferase fusion DNA (5 µg) and LacZ gene driven by SV40 promoter-enhancer sequence (2 µg) were introduced to VSMCs by the DEAE Dextran method according to the manufacturer's instruction (Promega Corporation). After transfection, the cells were cultured in DMEM with 10% FBS for 24 hours. Then, VSMCs were preincubated with or without telmisartan (10 µmol/L) for 60 minutes and stimulated with 10 ng/mL of TNFα in DMEM with 0.1% BSA. After 24 hours, the cells were lysed in 200 µL of Reporter lysis buffer (Promega Corporation). 100 µL of the lysate was used for luciferase activity assay in a Lumat luminometer (LB 9501, Berthold, Germany). Luciferase assay and β-galactosidase assay were performed as described previously. The luciferase activity was standardized by β-galactosidase activity.

Gel mobility shift assay
Cells were scraped off, washed in ice-cold PBS followed by ice-cold hypotonic buffer (buffer A: 10 mmol/L HEPES, pH7.9, 1.5 mmol/L MgCl₂, 10 mmol/L KCl, 0.5 mmol/L PMSF, 0.5 mmol/L DTT), and then lysed for 10 minutes on ice in the buffer A containing 0.1% Nonident P-40. The lysates were centrifuged for 10 minutes at 10000g. The pelleted nuclei were suspended in lysis buffer (20 mmol/L HEPES, pH7.9, 420 mmol/L NaCl, 1.5 mmol/L MgCl₂, 0.2 mmol/L EDTA, 25% glycerol, 0.5 mmol/L PMSF, 0.5 mmol/L DTT), incubated for 15 minutes at 4°C, and centrifuged for 10 minutes at 10000g. The supernatant was used as nuclear extracts.

Synthetic DNA probes (NF-kB: CAT GTG GGA TTT TCC CAT GA, C/EBPβ: CAC ATT GCA CAA TCT TAA) were labeled with ³²P by using [γ-³²P] ATP and T4 polynucleotide kinase, and purified by Sephadex G-50 column. Twenty µg of nuclear extracts was incubated with 1×10^5 cpm of labeled DNA probe and 2µg of poly (dI-dC) in a buffer containing 10 mmol/L Tris-HCl, pH7.5, 1 mmol/L EDTA, 4% glycerol, 100 mmol/L NaCl, 2.5 mmol/L DTT, 100mg/L bovine serum albumin for 15 minutes at room temperature. Then the samples were electrophoresed on 5% acrylamide/0.25×TBE gel (1×TBE 90 mmol/L of tris borate, 2 mmol/L of EDTA). After electrophoresis, gels were dried and exposed to x-ray film at -70°C.

Reverse transcription-polymerase chain reaction (RT-PCR)
Total RNA was prepared according to acid guanidinium thiocyanate-phenol-chloroform
Supplemental File

Qingping Tian et al

Then, the total RNA (0.4μg) was reverse-transcribed (RT) using molony murine leukemia virus reverse transcriptase (ReverTra Ace-α kit, TOYOBO). Semi-quantitative polymerase chain reaction (PCR) was performed with a T3000 Thermocycler (Biometra) according to the manufacturer’s instruction. An aliquot of RT-reaction mixture (0.5μL for amplification of TNF-Receptor 1(TNFR1) and 0.2μL for amplification of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) was subjected to PCR. The sequences for sense and antisense primers for TNFR1 were 5’- GAC TCA GGT ACT GCC GTG CT-3’ and 5’- CAT CCA CCA CAG CAT ACA GC-3’, respectively. GAPDH was used as a reference for the amount of cDNA. The sequences for sense and antisense primers for GAPDH were 5’-TTC TTG TGC AGT GCC AGC CTC GTC -3’ and 5’-TAG GAA CAG GGA AGG CCA TGC CAG-3’, respectively. Appropriate cycles for TNFR1 and GAPDH were determined to confirm the linear amplification of cDNA by PCR (supplementary figure IA). Twenty-eight cycles for TNFR1 and 22 cycles for GAPDH were used. The cDNAs of TNFR1 and GAPDH after PCR reaction were electrophoresed on 2% agarose gel, and stained with ethidium bromide. The density of TNFR1 and GAPDH cDNA visualized by ultraviolet transillumination was quantified with Image Gauge Softwear (Version 3.45).

Western blot analysis
VSMCs were lysed in a sample buffer (5 mmol/L EDTA, 10 mmol/L Tris-HCl, pH 7.6, 1% Triton X-100, 50 mmol/L NaCl, 30 mmol/L sodium phosphate, 50 mmol/L NaF, 1% aprotinin, 0.5% pepstatin A, 2 mmol/L phenylmethylsulfonyl fluoride and 5 mmol/L leupeptin). After electrophoresis on SDS-polyacrylamide gel, the protein was transferred to polyvinylidene fluoride membrane. Western blot analysis of MAPKs and α-tubulin was performed in a conventional method as described previously.2

Reference for supplemental methods

Supplemental Figures

Supplementary Fig. 1

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Supplementary Fig. 2

(A) pERK

(B) pP38
Supplementary Fig. 2

(C) 

![Image of Western Blot and Bar Graph showing protein expression levels of JNK phosphorylation]
Supplemental File
Qingping Tian et al

Legend for supplemental figures

Supplementary Figure 1. Effect of telmisartan on TNFR1 mRNA expression.
(A) PCR with different number of reaction cycles was performed to determine the linear amplification of the PCR products. Twenty eight cycles for TNFR1 and 22 cycles for GAPDH were chosen. The same result was obtained in other experiments (n=3).
(B) VSMCs were incubated with telmisartan (Tel: 10 µmol /L) for 1 hour or 24 hours. Then total RNA was prepared and expression of TNFR1 and GAPDH was determined by RT-PCR analysis. The densitometric analysis showed that telmisartan did not affect TNFR1 mRNA expression compared with unstimulated control (the number of independent experiments=3). We could not detect TNFR2 mRNA in our VSMC. No band was observed in samples without reverse transcription step for TNFR1 and GAPDH (data not shown).

Supplementary Figure 2. Effect of telmisartan on TNFα-induced MAPK activation.
VSMCs were stimulated with TNFα (10ng/ml) for 10 minutes in the presence or absence of telmisartan (Tel: 10µmol /L). Western blot analyses for total and phosphorylated forms of (A) ERK, (B) p38MAPK and (C) JNK were performed. Bar graphs indicated densitometric analysis. *p<0.05 vs. control. (the number of independent experiments=3) Telmisartan slightly inhibited TNFα-induced ERK and JNK phosphorylation. However, the difference was not statistically significant.