demonstrated that excess stimulation of cardiopulmonary and arterial baroreceptors impair baroreflex function[32,33] and RAS[32] or Rho-kinase[33] in the brain might contribute to the impaired baroreflex function. In the present study, we demonstrated that arterial pressure measured from the carotid artery and LVEDP were significantly greater in AB-4 mice than in Sham-4 mice. The excess stimulation of cardiopulmonary and arterial baroreceptor may contribute to the activation of the Rho/Rho-kinase pathway and RAS in the brains of the mice with pressure overload, even before high-salt loading.

In conclusion, the present findings strongly suggest that mice with pressure overload acquire brain Na sensitivity because of the activation of brain ENaCs via the Rho/Rho-kinase pathway and RAS. The acquired brain Na sensitivity contributes to high salt–induced sympathetic activation, leading to deteriorating cardiac function in mice with pressure overload.

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Disclosures

None.

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Online Data Supplement

Expanded Materials and Methods

Animals

The study was reviewed and approved by the Committee on Ethics of Animal Experiments, Kyushu University Graduate School of Medical Sciences, and conducted according to the Guidelines for Animal Experiments of Kyushu University. Male Institute of Cancer Research (ICR) mice (10 weeks old; SLC, Fukuoka, Japan) were used.

Mouse LVH Model Preparation

The suprarenal abdominal aorta[1] was banded in mice (AB mice) under sodium pentobarbital (25–40 mg/kg i.p.) anesthesia. The abdominal aorta was constricted at the suprarenal level with 5-0 silk sutures guided by a blunted 27-gauge needle, which was withdrawn as quickly as possible. Sham-operated (Sham) mice served as controls. Four weeks later, AB and Sham mice were each divided into 2 groups: 1) mice fed a high-salt (8% NaCl) diet for 4 weeks (AB-H mice and Sham-H mice) and 2) mice fed a regular-salt (0.3% NaCl) diet for 4 weeks (AB-R mice and Sham-R mice; Figure 1; protocol-1).

Evaluation of Cardiac Function

Cardiac function was evaluated by echocardiography.[2,3] Serial M-mode echocardiography was performed on mice under light sodium pentobarbital anesthesia with spontaneous respiration. An echocardiography system (SSD5000; Aloka, Tokyo, Japan) with a dynamically focused 7.5-MHz linear array transducer was used. M-mode tracings were recorded from the short-axis view at the

level of the papillary muscle. Left ventricle (LV) end-diastolic diameter (LVDD), LV end-systolic diameter (LVSD), and LV wall thickness (LVWT) were measured. LVWT was calculated as the average of the thickness of the interventricular septum and the posterior LV wall. Percent fractional shortening (%FS) was calculated as follows: %FS = (LVDD) − (LVSD) / (LVDD) × 100. Cardiac function was also evaluated by LV End-Diastolic Pressure (LVEDP). LVEDP was measured with a conductance catheter (1.4 Fr; Miller Instruments®) inserted into the right carotid artery and advanced across the aortic valve into the left ventricle.

Measurement of Arterial Pressure and Heart Rate

Under sodium pentobarbital anesthesia (25–40 mg/kg i.p.), mice were intubated using a 20-gauge soft catheter and ventilated with a tidal volume of 1.0-1.5 mL at 120 cycles/min with the fraction of inspired oxygen equal to 0.21.[2,4] A catheter was then inserted into the right carotid artery to measure arterial pressure and heart rate. In another protocol, arterial pressure and heart rate were measured in awake AB-H and AB-R mice using a radio-telemetry system (Data Sciences International).[5] Under sodium pentobarbital anesthesia (25–40 mg/kg i.p.), the telemetry catheter was implanted into the left carotid artery and the transducer unit was inserted into a subcutaneous pouch along the abdomen. Each mouse was housed in an individual cage after operation and unrestricted and free move in their cage. The case was placed over the receiver panel connected to the computer for data acquisition. Arterial pressure and heart rate were recorded continuously for 5 minutes and averaged.

Evaluation of Sympathetic Activity

Sympathetic activity was evaluated by measuring 24-h urinary norepinephrine (U-NE) and urinary epinephrine (U-E) excretion using high-performance liquid chromatography.[2,4]

Evaluation of Na Sensitivity

U-NE and U-E after high salt intake was compared between Sham mice and AB mice. Four weeks after AB (AB-4 mice) or sham operation (Sham-4 mice), mice were fed a high-salt (8% NaCl) diet. Five days after starting the high-salt diet, 24-h U-NE and U-E excretion were measured, and echocardiography was performed to confirm that cardiac function was preserved. In addition, U-NE and U-E excretion in response to high-Na (0.2 mol/L) aCSF ICV infusion (0.25 L/h for 14 days, using an osmotic minipump) were measured in Sham mice and AB mice. Under anesthesia with sodium pentobarbital (25–40 mg/kg i.p.), mice were placed on a stereotaxic frame. The skin overlying the midline of the skull was incised, and a small hole with the following coordinates was bored using a dental drill: 0.3 mm posterior and 1 mm lateral relative to the bregma, and 3 mm ventral to the skull surface.[6] An Alzet® brain infusion kit 3 (DURECT Corporation, CA) connected to an osmotic minipump (Alzet model 1004; DURECT) was fixed to the skull surface with tissue adhesive. The pump was inserted subcutaneously on the back. Mice with ICV infusion of regular-Na (0.145 mol/L) aCSF served as the controls (R-Na ICV-mice; Figure 1; protocol-2). Before and 2 weeks after starting the ICV infusion, sympathetic activity, cardiac function, arterial pressure, and heart rate were measured by the methods described above.

The effects of high-Na (0.2 mol/L) aCSF ICV infusion on arterial pressure and heart rate were also evaluated in Sham-4 mice and AB-4 mice in acute experiments. Arterial pressure and heart rate were measured via a catheter in the right carotid artery under anesthesia. High-Na (0.2 mol/L) aCSF was infused ICV with a microsyringe pump (infusion rate: 1 L/min for 10 min) and changes in arterial pressure and heart rate were measured. Furthermore, the effects of other central stimuli, such as angiotensin II or carbachol ICV infusion on arterial pressure were

examined. Angiotensin II (Sigma) (0.5 nmol/L, 1 L/min for 5 min) or carbachol (Sigma) (0.1 mmol/L, 1 L/min for 5 min) was infused. The dose of each chemical was also determined according to the previous reports.[7,8]

Measurement of Brain Na Concentration

Under anesthesia with an overdose of sodium pentobarbital, the mice were perfused with dH_2O. After adequate perfusion to remove blood, the brain circumventricular tissues and hypothalamus were dissected out. The tissues (0.10 ± 0.01 g) were homogenized in 200 L of dH_2O, centrifuged, and the supernatant was collected. The Na concentration in each sample was measured.

Measurement of Organ Weight

After completion of the experiments, mice were killed with an overdose of sodium pentobarbital, and the heart and lungs were removed and weighed.

Measurement of Serum Parameters

Within minutes after the mice were injected with an overdose of sodium pentobarbital, a blood sample was collected from the right ventricle and rapidly centrifuged (6000 rpm for 10 min). The obtained serum sample was then stored at −20°C before measuring serum components. We evaluated the aldosterone concentration by radioimmunoassay, Na concentration by electrode methods, and creatinine by enzymatic methods.

Evaluation of the Effects of Na-Channel Blockade in the Brain

Benzamil (Sigma), a specific epithelial Na-channel (ENaC) blocker[9] (1 mg/mL, dissolved in

aCSF), was infused ICV in AB-H mice (AB-HB mice) and AB-R mice (AB-RB mice) using an osmotic minipump (0.11 L/h for 4 weeks). Four weeks later, 24-h U-NE and U-E excretion, arterial pressure, heart rate, and organ weight were measured, and echocardiography was performed as described earlier. Mice with ICV infusion of only aCSF (vehicle) served as controls (aCSF mice; Figure 1; protocol-1).

Evaluation of the Effects of Rho-Kinase and Angiotensin Type 1 Receptors (AT1R) Blockade in the Brain

A specific Rho-kinase inhibitor, Y-27632[10] (Calbiochem, 5 mmol/L, dissolved in aCSF), or an AT1R blocker, telmisartan (Sigma, 4 mmol/L, 20 mmol/L, dissolved in demethyl sulfoxide [DMSO]) was infused ICV in ABH mice (AB-HY or AB-HT mice, respectively) using an osmotic minipump (0.11 L/h for 4 weeks). Four weeks later, 24-h U-NE/U-E excretion, arterial pressure, heart rate, and organ weight were measured; echocardiography was performed in AB-HY mice and AB-HT mice as described earlier (Figure 1; protocol-1).

Evaluation of AT1R Expression and Rho-Kinase Activity

The animals were killed with an overdose of sodium pentobarbital, and circumventricular tissues including the hypothalamus and brainstem tissues were obtained. The tissues were homogenized in a lysing buffer containing 40 mmol/L HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid), 1% Triton® X-100, 10% glycerol, 1 mmol/L Na_3VO_4 (sodium orthovanadate), and 1 mmol/L phenylmethylsulfonyl fluoride. The tissue lysate was centrifuged and the supernatant collected. Protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL). A 15- g aliquot of protein from each sample was separated on a polyacrylamide gel with 10% sodium dodecyl sulfate. The proteins were subsequently

transferred onto polyvinylidene difluoride membranes (Immobilon®-P membranes; Millipore, Billerica, MA). Membranes were incubated with rabbit immunoglobulin G (IgG) monoclonal antibody to angiotensin type-1 receptor (AT1Rs, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), with rabbit IgG polyclonal antibody to β-tubulin (1:1000; Santa Cruz Biotechnology) and with goat IgG polyclonal antibody to phosphorylated-moesin, a substrate of Rho-kinase[11] (p-moesin, 1:1000, Santa Cruz Biotechnology). Membranes were then incubated with horseradish peroxidase-conjugated horse anti-rabbit or anti-goat IgG antibody (1:10,000). β-Tubulin (1:5000; Santa Cruz Biotechnology) was used as an internal control for the brain tissues. Immunoreactivity was detected by enhanced chemiluminescence autoradiography (ECL™ Western blotting detection kit; Amersham Pharmacia Biotech, Uppsala, Sweden), and the film was analyzed using the public domain software NIH Image (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Statistical Analysis

All values are expressed as mean ± SE. Analysis of variance was used to compare U-NE and U-E excretion, organ weight, LVDD, LVWT, %FS, and arterial pressure by telemetry system between groups. An unpaired *t*-test was used to compare changes in arterial pressure and heart rate after high-Na ICV infusion, and protein levels between Sham mice and AB mice. Differences were considered to be significant when $P < 0.05$.

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Online Table I **LVEDP for Each Group**

Group	LVEDP (mmHg)
Sham- 4	7.0 ± 0.8
Sham-R	9.6 ± 0.2
AB-4	13.5 ± 1.6 *
AB-R	13.3 ± 1.1
AB-H	18.6 ± 0.8 #

n=4 for each, *P<0.05 versus Sham-4, #P<0.05 versus Sham-R

Online Table II MAP and HR for Each Group

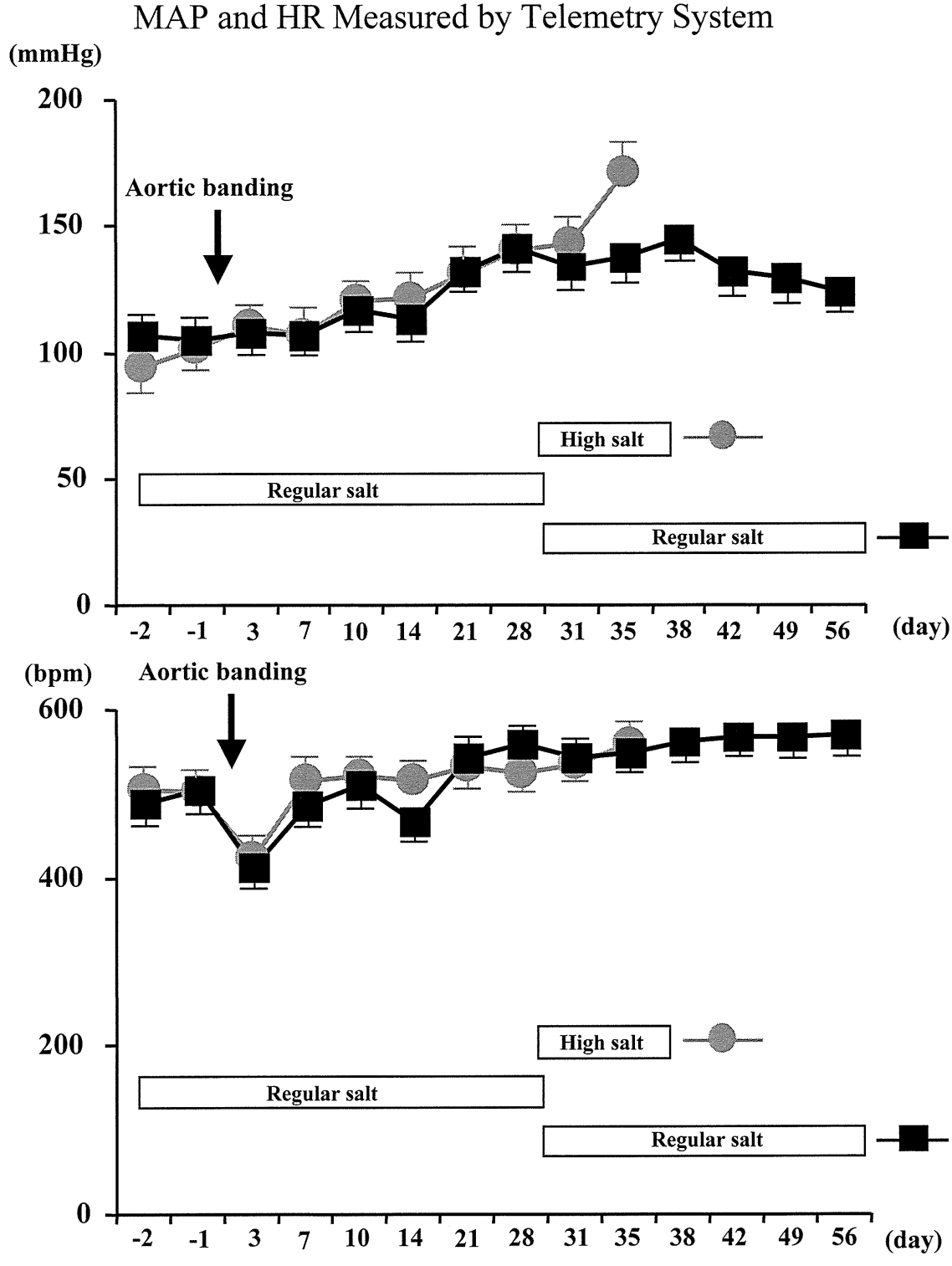
Group (Number)	MAP (mmHg)	HR (bpm)
Sham- 4 (8)	87 ± 2	388 ± 9
Sham-R (5)	88 ± 2	390 ± 5
Sham-H (5)	95 ± 3	401 ± 6
AB-4 (8)	107 ± 4 *	429 ± 28 *
AB-R (5)	94 ± 3	460 ± 14 $^{\#}$
AB-H (5)	80 ± 5	497 ± 3 $^{\#,+}$
AB-H 1w (5)	123 ± 7	490 ± 15 *
AB-HB (4)	95 ± 5	435 ± 17 $^{\$}$
AB-HY (5)	94 ± 3	372 ± 15 $^{\$}$
AB-HT (4)	88 ± 1	402 ± 6 $^{\$}$
AB-4 (FA) (6)	92 ± 4	

*$P<0.05$ versus Sham-4, $^{\#}P<0.05$ versus Sham-R, $^{+}P<0.05$ versus AB-R

$^{\$}P<0.05$ versus AB-H

FA measured from femoral artery.

Online Figure I



Online Figure I: Graphs showing mean arterial pressure (MAP) (upper panel) and heart rate (HR) (lower panel) measured by telemetry before and after aortic banding (arrow). Circles indicate the data from mice fed a high salt diet and squares indicate the data from mice fed a regular salt diet. Please see details in the Results section of the text.

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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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: 798-804.)**

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

Angiotensin II (Ang II) is a main final effecter molecule of the renin–angiotensin system. Physiologically, Ang II plays an important role in the regulation of blood pressure, fluid volume, and electrolyte balance.[1] However, Ang II is also involved in the pathological processes, such as cardiovascular diseases, renal insufficiency, and metabolic disorders.[2] Indeed, inhibition of the renin–angiotensin system by Ang II type 1 receptor (AT1R) antagonists has been proven beneficial for treatment of heart failure,[3] chronic kidney diseases,[4] and myocardial infarction.[5] AT1R antagonists also showed favorable effects on prevention of new onset of diabetes mellitus and atrial fibrillation.[6,7]

Telmisartan, one of the AT1R antagonists, was reported to be a partial agonist of peroxisome proliferator-activated receptor-γ (PPARγ).[8,9] PPARγ is a nuclear receptor transcription factor,[10] 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.[11] 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.[12]

Interleukin-6 (IL-6) is one of the proinflammatory cytokines and is induced by tumor necrosis factor-α (TNF-α),[13] Ang II,[14] and other stimuli in vascular smooth muscle cells (VSMCs), endothelial cells, and macrophages. IL-6 plays an

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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. [α-^{32}P] dCTP and [γ-^{32}P]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 DMEM with 0.1% BSA, and Ang II was suspended in sterile water. 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.

Cell Culture

VSMCs were isolated from the thoracic aorta of Sprague–Dawley rats and cultured in a humidified atmosphere of 95% air/5% CO_2 at 37°C in DMEM as described previously.[14] Cells were grown to confluence and growth-arrested in DMEM with 0.1% BSA for 2 days before use. Passages between 5 and 13 were used for the experiments.

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-κB–luciferase and CCAAT/enhancer-binding protein-β (C/EBPβ)–luciferase were purchased from Stratagene Co. Five copies of NF-κB 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 previously[14] using synthetic NF-κB and C/EBPβ DNA probe (NF-κB: 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 C57/BL6 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

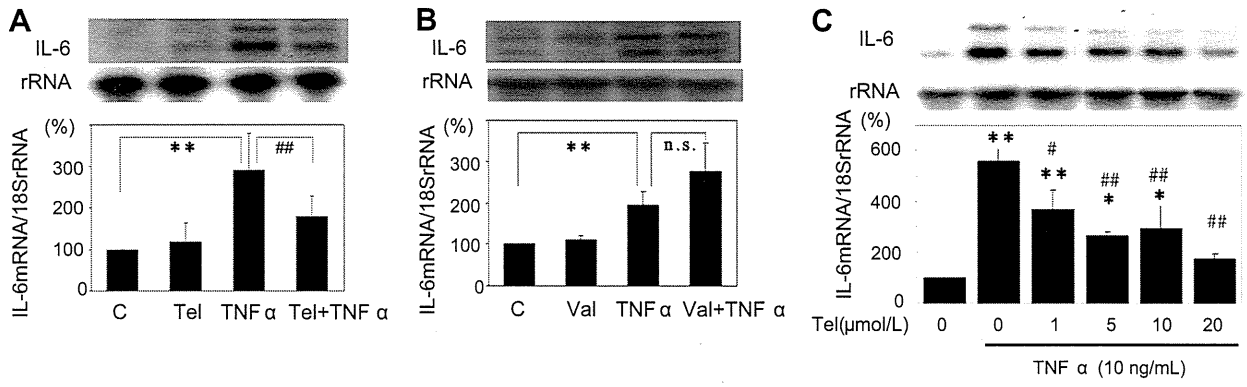

Figure 1. Suppression of TNF-α–induced IL-6 mRNA expression by telmisartan (Tel). VSMCs were preincubated with Tel (10 μmol/L; A), valsartan (Val; 10 μmol/L; B), or various concentrations (1 to 20 μmol/L; C) of telmisartan for 60 minutes and stimulated with TNF-α (10 ng/mL) for 30 minutes. Total RNA 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-α.

clinical study[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 PPARα.[9] 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-α (10 ng/mL) with or without preincubation with telmisartan (1 to 20 μmol/L). TNF-α–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 lower concentrations (Figure 2B); thus, we confirmed that telmisartan is an effective AT1R antagonist.

We next 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 IB). We could not detect TNF type 2 receptor mRNA in our VSMCs. We also examined the effect of telmisartan on TNF-α–induced MAPK activation (supplemental Figure II). Telmisartan did not affect TNF-α–induced activation of ERK, p38MAPK, or JNK.

Telmisartan Inhibition of TNF-α–Induced IL-6 Expression Was Dependent on PPARγ

To clarify the role of PPARγ in telmisartan inhibition of TNF-α–induced IL-6 expression, the effect of GW9662, a PPARγ-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-α–induced IL-6 expression (Figure 3A). Pioglitazone (10 μmol/L; preincubation for 1 hour), a full PPARγ agonist, also suppressed the TNF-α–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-α (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

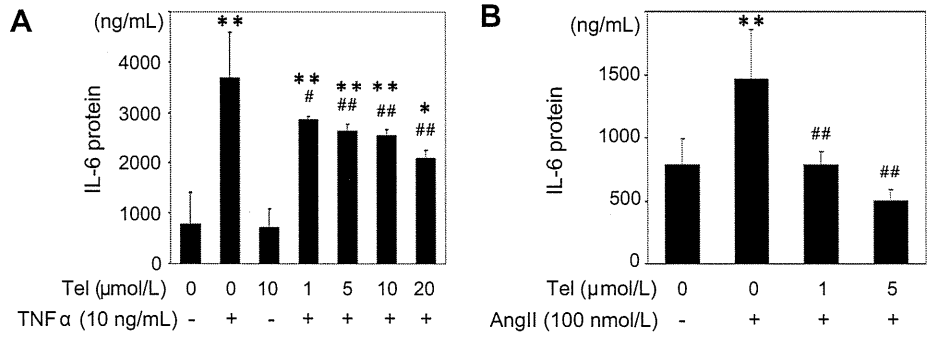

Figure 2. Suppression of TNF-α– and Ang II–induced IL-6 protein production by telmisartan (Tel). A, VSMCs were preincubated with Tel (10 μmol/L) at various concentrations for 60 minutes and stimulated with TNF-α (10 ng/mL) for 24 hours. B, VSMCs were incubated with Tel at 1 or 5 μmol/L and stimulated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

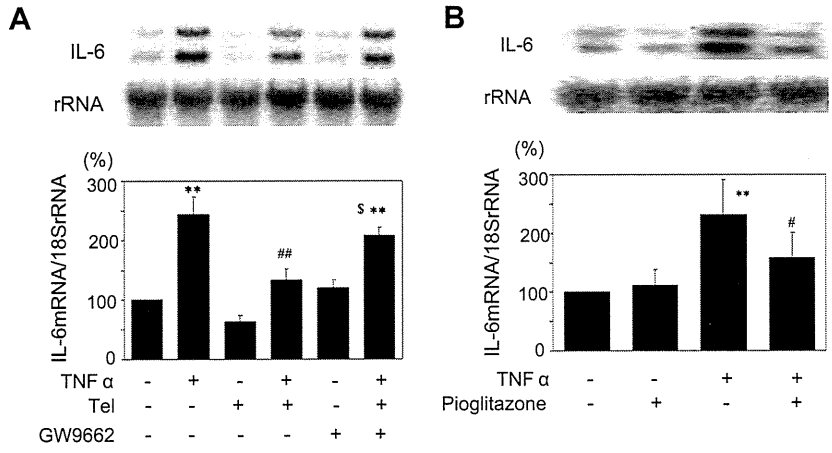

Figure 3. Effect of GW9662 on telmisartan (Tel) inhibition of TNF-α–induced IL-6 expression. A, VSMCs were incubated with GW9662 (10 μmol/L) for 3 hours followed by preincubation with Tel (10 μmol/L) for 60 minutes. Then the VSMCs were stimulated with TNF-α (10 ng/mL) for 30 minutes. B, VSMCs were preincubated with pioglitazone (10 μmol/L) for 60 minutes, then stimulated with TNF-α (10 ng/mL) for 30 minutes. Northern blot analysis of IL-6 mRNA was performed as described in Figure 1 legend. **$P<0.01$ vs control; #$P<0.05$ vs TNF-α; ## $P<0.01$ vs TNF-α; \$$P<0.05$ vs Tel+TNF-α (No. of independent experiments was 4).

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.[20] 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). To

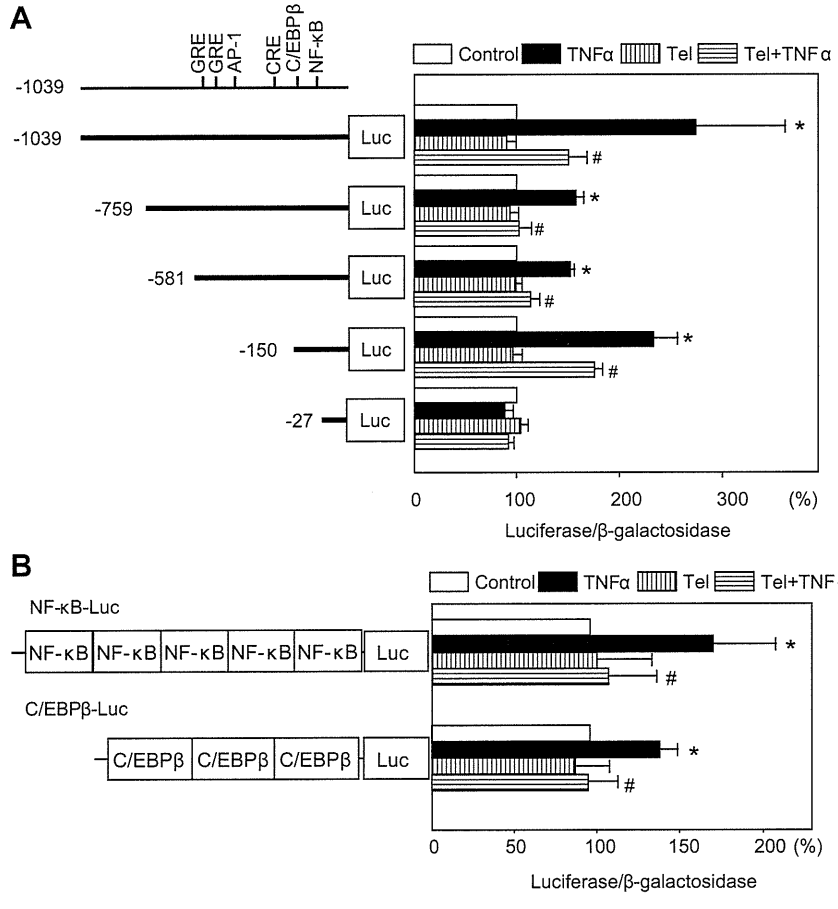

Figure 4. Suppression of IL-6 gene promoter activity by telmisartan (Tel). A, After transfection of IL-6 gene promoter/luciferase (Luc) fusion DNA (5 μg), VSMCs were preincubated with or without Tel (10 μmol/L; 60 minutes) and stimulated with TNF-α (10 ng/mL) for 24 hours. AP-1 indicates activator protein-1. B, NF-κB–Luc or C/EBPβ–Luc was introduced to VSMCs. VSMCs were preincubated with or without Tel (10 μmol/L; 60 minutes) and stimulated with TNF-α (10 ng/mL) for 24 hours. Luc activity was normalized with β-galactosidase activity. The relative promoter activity without stimulation (control) was set as 100%. *$P<0.01$ vs control; #$P<0.05$ vs TNF-α (No. of independent experiments was 4).

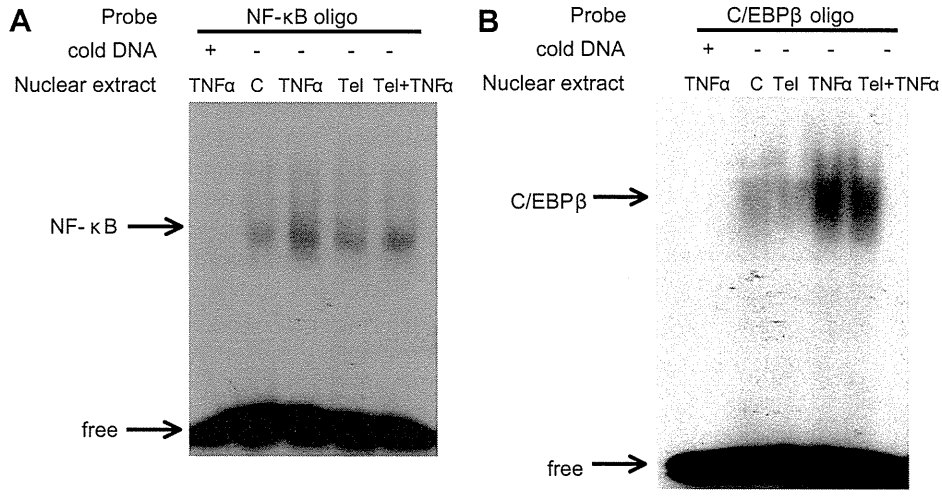

Figure 5. Telmisartan (Tel) attenuated TNF-α–induced NF-κB and C/EBPβ binding. A, Binding activity of NF-κB sequence of IL-6 gene promoter to nuclear extracts from unstimulated (C), TNF-α–stimulated, Tel-stimulated, and Tel- and TNF-α–stimulated VSMCs were examined by gel mobility shift assay. B, Binding activity of C/EBPβ sequence of IL-6 gene promoter to nuclear extracts from unstimulated, Tel-stimulated, TNF-α–stimulated, and Tel- and TNF-α–stimulated VSMCs were examined by gel mobility shift assay. Fifty times molar excess of unlabeled oligonucleotide (oligo) was added to the reaction mixture in the left lane (Cold DNA+). The same results were obtained in other independent experiments (No. of independent experiment was 3).

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 stimulus. 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.[22] 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.[12] 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.

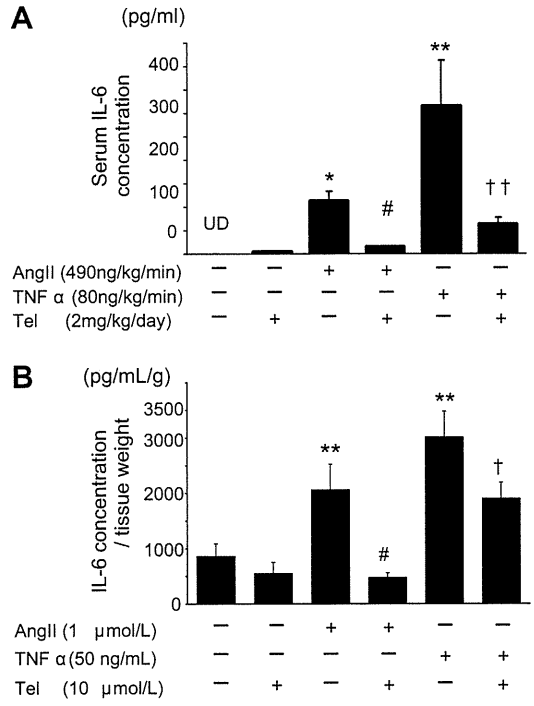

Figure 6. Telmisartan attenuated IL-6 production in vivo and ex vivo. A, Serum concentration of IL-6 was measured in mice injected with Ang II (490 ng/kg per minute) or TNF-α (80 ng/kg per minute) in the presence or absence of telmisartan (Tel; 2 mg/kg per day) administration for 1 week (No. of independent experiments was 5). *P<0.05; **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.

tion of TNF-α–induced NF-κB DNA binding. In contrast, activated PPARγ 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.[26] IL-6 enhanced VSMC growth induced by platelet-derived growth factor.[27] IL-6 also increased both monocyte chemoattractant protein-1 production and DNA synthesis of VSMCs, which may coordinate inflammatory and proliferative responses.[28] IL-6 is also a useful biomarker predicting future cardiovascular events.[29] 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.[30] 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.[31] Another report showed that C/EBPβ regulated monocyte chemoattractant protein-1 expression in the aorta of hyperinsulinemic rats.[32] 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,[33] 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.[11] Rosiglitazone was also shown to have additive effects on plaque regression in the combination treatment with simvastatin in an atherosclerotic rabbit model.[34] AT1R antagonists were also reported to suppress atherogenesis. Strawn et al demonstrated that losartan attenuated atherogenesis in monkeys with hypercholesterolemia.[35] Based on these studies and our results, telmisartan may be more protective against vascular lesion formation attributable to PPARγ 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 PPARγ. 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.

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Disclosures

None.

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Soluble Flt-1 Gene Transfer Ameliorates Neointima Formation After Wire Injury in flt-1 Tyrosine Kinase Deficient Mice

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