Biochemical and Biophysical Research Communications 454 (2014) 320–327

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Telmisartan, a possible PPAR-δ agonist, reduces TNF-α-stimulated VEGF-C production by inhibiting the p38MAPK/HSP27 pathway in human proximal renal tubular cells

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ARTICLE INFO

Article history:
Received 4 October 2014
Available online 22 October 2014

Keywords:
VEGF-C
p38MAPK
HSP27
TNF-α
Telmisartan
PPAR

ABSTRACT

Vascular endothelial growth factor-C (VEGF-C) is a main inducer of inflammation-associated lymphangiogenesis in various inflammatory disorders including chronic progressive kidney diseases, for which angiotensin II receptor type 1 blockers (ARBs) are widely used as the main treatment. Although proximal renal tubular cells may affect the formation of lymphatic vessels in the interstitial area by producing VEGF-C, the molecular mechanisms of VEGF-C production and its manipulation by ARB have not yet been examined in human proximal renal tubular epithelial cells (HPTECs).

In the present study, TNF-α dose-dependently induced the production of VEGF-C in HPTECs. The TNF-α-induced production of VEGF-C was mediated by the phosphorylation of p38MAPK and HSP27, but not by that of ERK or NFkB. Telmisartan, an ARB that can activate the peroxisome proliferator-activated receptor (PPAR), served as a PPAR-δ activator and reduced the TNF-α-stimulated production of VEGF-C. This reduction was partially attributed to a PPAR-δ-dependent decrease in p38MAPK phosphorylation.

Our results indicate that TNF-α induced the production of VEGF-C in HPTECs by activating p38MAPK/HSP27, and this was partially inhibited by telmisartan in a PPAR-δ dependent manner. These results provide a novel insight into inflammation-associated lymphangiogenesis.

1. Introduction

Vascular endothelial growth factor (VEGF)-C belongs to the VEGF family of growth factors. VEGF-C is synthesized as a 44 kDa precursor, proteolytically cleaved, and then secreted as a 20 kDa mature protein [1]. Processed VEGF-C can bind to VEGF receptors 2 and 3. Previous studies demonstrated that VEGF-C served as a more potent inducer of lymphangiogenesis via VEGF receptor 3 on the lymphatic endothelium [2,3]. The expression of VEGF-C was shown to be high in perinephric, mesenteric, and cephalic regions during embryonic development, but was lower in adult tissues including lymph nodes, the heart, and intestines [3].

Recent studies showed that lymphatic neo-angiogenesis was enhanced in several pathological conditions including progressive kidney diseases [4–6]. Increases in newly formed lymphatic vessels have been reported in chronic inflammatory lung diseases [4] and after myocardial infarction [5]. An increase in lymphatic neo-angiogenesis was most recently observed in inflammatory and fibrotic lesions in the tubulointerstitium of various human kidney diseases [6] as well as obstructive nephropathy in rodents [7,8]. Lymphatic growth was positively associated with the expression of VEGF-C in inflammatory mononuclear cells and proximal renal tubular cells [6–8].

Previous studies using human [9] and mouse cells [10] analyzed the molecular mechanisms underlying the production of VEGF-C. Inflammatory cytokines (TNF-α and IL-1β) [9,10] upregulated the expression of VEGF-C. This induction of VEGF-C was shown to be mediated via the activation of p38MAPK [11] or NFkB [10,11]. A recent study showed that high concentrations of sodium chloride increased the expression of VEGF-C by activating a tonicity-response

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http://dx.doi.org/10.1016/j.bbrc.2014.10.077
0006-291X/

enhancer binding protein in mouse macrophages [12]. Although renal proximal tubular cell lines have also been shown to produce VEGF-C [6–8], the mechanisms underlying the production of VEGF-C by human proximal renal tubular epithelial cells (HPTECs) have not yet been examined in detail.

Angiotensin II (Ang II) receptor blockers (ARBs) are used worldwide to treat chronic progressive kidney disease in which chronic and/or acute inflammation occurs due to underlying pathological conditions. By blocking type 1 Ang II receptors, ARBs not only ameliorate hypertension, but also inhibit the Ang II-stimulated induction of pro-fibrotic cytokines and oxidative stress, thereby retarding the progression of chronic renal parenchymal injury [13,14]. Some ARBs have also been shown to act as peroxisome proliferator-activated receptor (PPAR) activators [15] and consequently induce anti-inflammatory effects by interfering with inflammatory signaling [16]. One unique ARB, telmisartan, has recently been attracting attention due to its putative PPAR-γ [17,18] or δ actions [19,20] and ensuing anti-inflammatory effects [21,22]. In view of these anti-inflammatory actions, ARBs may affect the expression of VEGF-C and lymphangiogenesis in the damaged kidney. However, the effects of ARBs on the production of VEGF-C in renal resident cells currently remain unknown.

In the present study, we attempted to clarify the molecular mechanism underlying the production of VEGF-C and its modulation by telmisartan, as a possible PPAR modulator, as well as ARBs in HPTECs.

2. Materials and methods

2.1. Materials

SB203580, GW501516 (Enzo Life Sciences Plymouth Meeting, PA, USA), H89 (LC Laboratories, Boston, MA, USA), telmisartan, pioglitazone, GW9662, GSK0660 (SIGMA–Aldrich, St. Louis, MO, USA), PD98059, and Nuclear factor-kB (NF-kB) inhibitor (Merck KGaA, Darmstadt, Germany) were used. A goat polyclonal antibody against human phosphorylated extracellular signal-regulated kinase 1/2 (pERK1/2; Thr202/Tyr204), mouse monoclonal antibodies against human phosphorylated p38MAPK (p-p38MAPK; Thr180/Tyr182), human HSP27, and human nuclear factor-kB (NFkB), and rabbit monoclonal antibodies against human phosphorylated NFkB (Ser536), human cAMP response element-binding protein (CREB), and human phosphorylated CREB (pCREB; Ser133) were purchased from Cell Signaling Technology. Rabbit polyclonal antibodies against human PPAR-γ (Santa Cruz Biotechnology, CA, USA), human ERK1/2, p38 mitogen-activated protein kinase (p38MAPK), human phosphorylated heat shock protein 27 (pHSP27; Ser82) (Cell Signaling Technology, Boston, USA), and human β-actin (Abcam, Cambridge, UK) were used. A mouse monoclonal antibody against human PPAR-δ (Santa Cruz Biotechnology) and horseradish peroxidase (HRP)-conjugated anti-rabbit, anti-goat, and anti-mouse immunoglobulins (Dako, Glostrup, Denmark) were also used. The Dual-Luciferase Reporter Assay System was purchased from Promega (Madison, WI, USA). Human recombinant TNF-α was purchased from PeproTech (Rocky Hill, NJ, USA).

2.2. Tubular cell cultures

Two origin-different HPTECs (Lot No. 7F4202, 109950) were purchased as twice-passaged cells from Lonza Walkersville, Inc. (Walkersville, MD, USA). These cells were grown in Renal Epithelial Cell Growth Medium (REGM) (Lonza Walkersville, Inc.) with 5% CO_2 and 95% air in a humidified atmosphere at 37.0 °C. The specific outgrowth of HPTECs was characterized as described previously [23]. HPTECs (passage 3 through 5) were seeded on 12-well plates. The REGM was renewed every 2 days until confluence was achieved. Confluent cells were growth-arrested in DMEM (Invitrogen Corp, Carlsbad, CA, USA) with 0.5% fetal bovine serum (FBS) (Invitrogen Corp.) for 24 h, and the DMEM was renewed immediately before the stimulation experiment. Human recombinant TNF-α was added to the medium at a final concentration of 10 ng/mL for 24 h unless otherwise stated. To identify signaling pathways involved in TNF-α-induced expression of VEGF-C, HPTECs were treated with SB203580, H89, PD95089, and NF-kB inhibitor for 30–60 min before the TNF-α treatment. SB203580, H89, PD95089, NF-kB inhibitor, telmisartan, GW9662, GSK0660, and pioglitazone were prepared as stock solutions in dimethyl sulfoxide (DMSO, SIGMA–Aldrich). These reagents were further diluted to working concentrations in the cell incubation medium. The final concentration of DMSO in our experiments did not exceed 0.1%.

2.3. Determination of human VEGF-C concentrations in supernatants

VEGF-C concentrations were determined in supernatants using a commercially available ELISA kit (Quantikine Human VEGF-C; R&D Systems Inc., Minneapolis, MN, USA).

2.4. TaqMan real-time PCR assay

The TaqMan real-time PCR assay was performed using the TaqMan ABI 7000 sequence Detection System (Applied Biosystems) as previously reported [24]. Unlabeled specific primers and TaqMan MGB probes (6-FAM dye-labeled) were purchased from Applied Biosystems to detect the human VEGF-C gene (Assay ID: Hs00167155_m1). The TaqMan human β-actin MGB (VIC dye-labeled) control reagent kit (Applied Biosystems, accession No: NM_001101) was used to detect human β-actin. VEGF-C mRNA amounts were normalized to β-actin mRNA levels.

2.5. Immunoblot analysis

HPTECs were lysed in RIPA buffer containing phosphatase inhibitors (SIGMA–Aldrich). Ten micrograms of protein was separated on an 8% SDS–polyacrylamide gel (PAGE) and then electrophoretically transferred to nitrocellulose membranes (Trans-Blot SD; BioRad, Hercules, CA, USA). The membranes were blocked with Blocking One or Blocking One-P (Nacalai Tesque, Kyoto, Japan) in Tris-buffered saline (pH 8.0) containing 0.05% Tween-20 (TBS-T) at 37 °C for 1 h and then incubated with anti-p38MAPK (1:1000 dilution), anti-p-p38MAPK (1:2000 dilution), anti-CREB (1:1000 dilution), anti-pCREB (1:1200 dilution), anti-ERK1/2 (1:100 dilution), anti-pERK1/2 (1:1000 dilution), anti-NFkB (1:1000 dilution), anti-pNFkB (1:1000 dilution), anti-HSP27 (1:1000 dilution), and anti-pHSP27 (1:1000 dilution) antibodies for 1 h at room temperature. The membranes were then incubated with appropriate horseradish peroxidase-conjugated anti-rabbit, anti-goat, and anti-mouse immunoglobulins (1:1000 dilution) at room temperature for 1 h. The detection of secondary antibodies was performed as described previously [24].

2.6. Transient transfection of a PPAR response element-luciferase expression vector

PPAR-induced transcriptional activity was evaluated via the transient transfection of an expression vector containing four copies of a consensus PPAR response element (PPRE), which was placed upstream of the TK-luciferase reporter (pPPRE-TK-Luc), as reported previously [25]. HPTECs were seeded on 24-well plates at a density of 5×10^4 cells/well and incubated in REGM without antibiotics. The next day, cells were transfected with 400 ng of the pRL-TK control vector or 400 ng of the

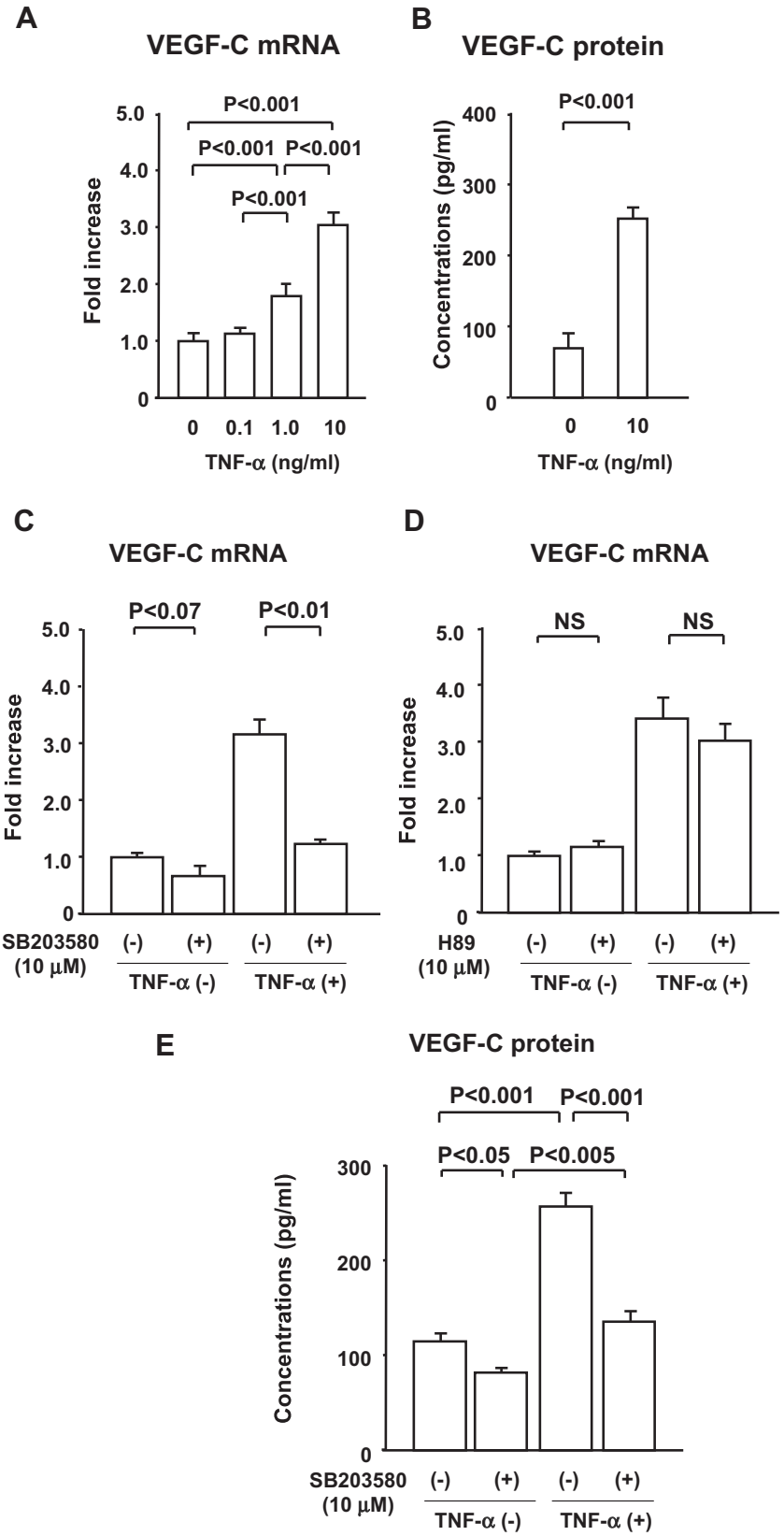

Fig. 1. TNF-α induced VEGF-C expression via the p38MAPK pathway. (A and B) HPTECs were treated with TNF-α (0.1, 1.0, or 10 ng/mL) for 24 h. (C–E) HPTECs were incubated for 24 h with DMEM containing no TNF-α or TNF-α (10 ng/mL) in the presence or absence of SB203580 (C) and H89 (D). VEGF-C mRNA amounts (C and D) and concentrations (E) were determined by real-time PCR assay and ELISA, respectively. Results are expressed as the mean ± SD of a representative experiment (n = 3–6). NS: not significant, and P values, significantly different from cells incubated under the indicated conditions, according to a one-way ANOVA with Scheffe's post hoc comparison (A and C–E) and the Mann–Whitney test (B).

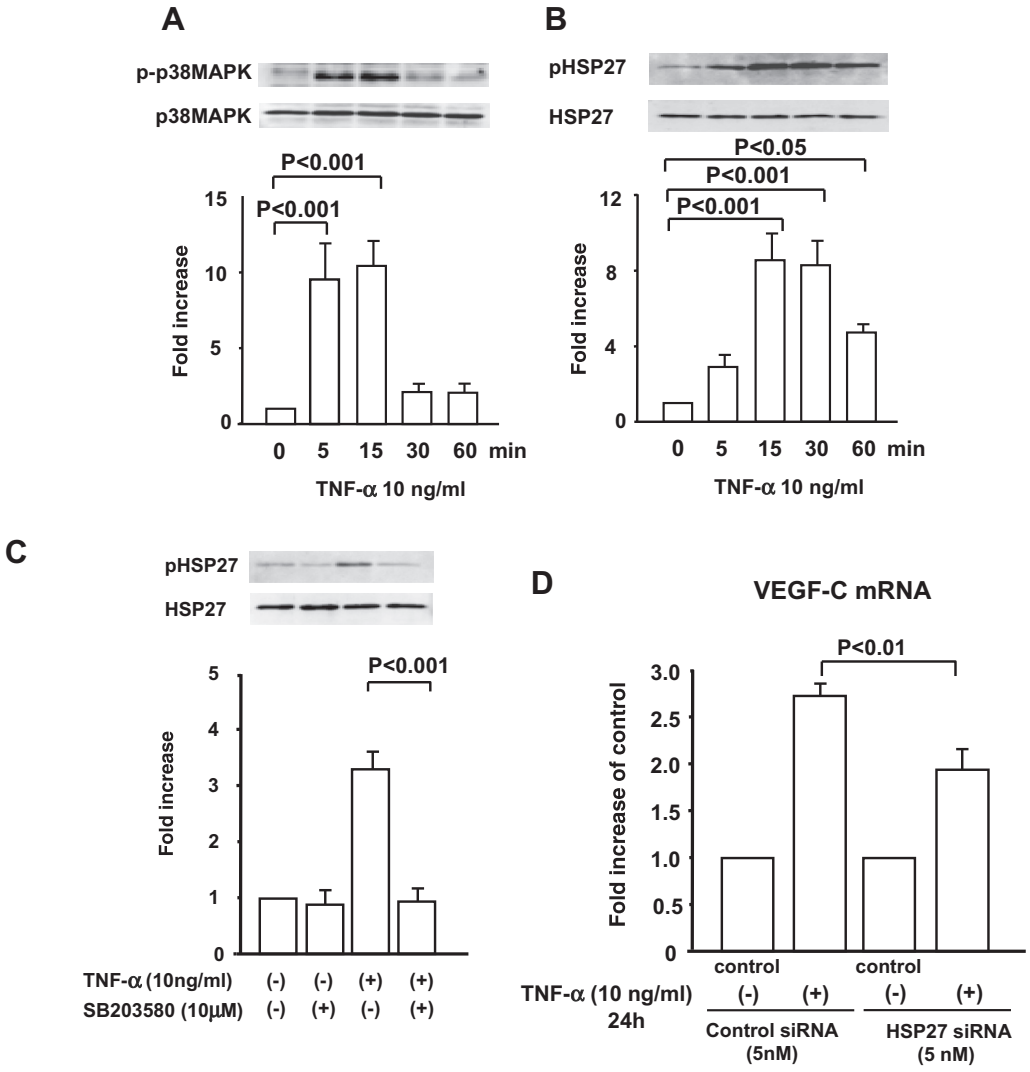

Fig. 2. TNF-α increased VEGF-C expression via the p38MAPK-enhanced phosphorylation of HSP27. (A and B) HPTECs were treated with TNF-α for 0, 5, 15, 30, and 60 min. (C) HPTECs were treated for 15 min with or without TNF-α in the presence or absence of SB203580. The amounts of p-p38MAPK (A) and pHSP27 (B and C) were determined by immunoblot analyses and normalized to p38MAPK and HSP27 levels, respectively. Representative blots are shown (upper panel). (D) VEGF-C mRNA amounts were determined in siRNA-mediated HSP27 knockdown cells and control cells treated with or without the TNF-α treatment. Results are expressed as the mean ± SD of three to four independent experiments. *P* values, significantly different from cells incubated under the indicated conditions, according to a one-way ANOVA with Scheffe's post hoc comparison (A–C) and the Mann–Whitney test (D).

pPPRE-TK-Luc using Lipofectamine 2000 (Invitrogen Corp.) and OPTI-MEN (Invitrogen Corp.). Four hours later, the transfection medium was replaced with DMEM containing 0.5% BSA and then placed in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C for 24 h. The DMEM was renewed immediately before the 24-h stimulation experiments. GW9662 and GSK0660 were added to the medium, 6 and 2 h before and during the stimulation treatment, respectively. Cells were lysed in lysis buffer, and firefly luciferase and renilla luciferase activities were determined in the lysates with a luminometer using the dual-luciferase reporter assay kit (Promega Corp., WI, USA). All experiments were performed at least three times, and data were normalized to renilla activity.

2.7. Transfection of small interfering RNA against HSP27

Small interfering RNAs (siRNA) against HSP27 and control siRNA (a non-targeting siRNA) were purchased from Thermo Scientific Dharmacon (Lafayette, CO, USA). HPTECs (70% confluence) were transfected with the negative control siRNA or siRNA against HSP27 at a final concentration of 5 nmol/L using the Lipofectamine RNAiMAX transfection reagent (Invitrogen), according to the manufacturer's instructions. After being incubated for 48 h, the HPTECs that had been refreshed with DMEM were treated with or without TNF-α for an additional 24 h and the expression of VEGF-C mRNA was then analyzed.

2.8. Statistical analyses

All data are presented as means plus standard deviation (±SD). The Mann–Whitney test was used to evaluate the significance of differences between two groups of experiments. A one-way analysis of covariance (ANOVA) with Scheffe's or Fisher's post hoc test was used for multiple comparisons. A two-tailed *P* value of less than 0.05 was considered to be significant.

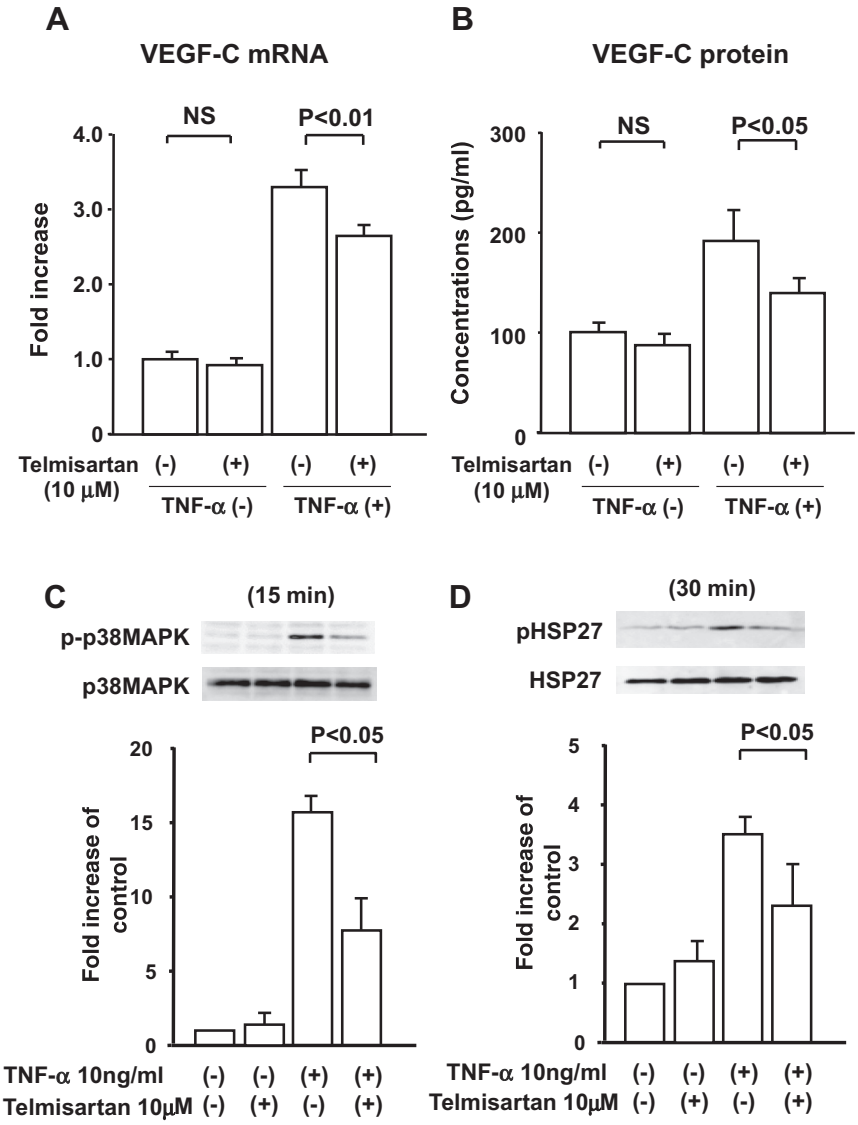

Fig. 3. Telmisartan decreased VEGF-C expression by reducing the phosphorylation of p38MAPK and HSP27. (A and B) HPTECs were treated for 24 h with or without TNF-α (10 ng/mL) in the presence or absence of telmisartan. VEGF-C mRNA levels and VEGF-C concentrations were determined by real-time PCR assays and ELISA, respectively. (C and D) HPTECs were treated with or without TNF-α in the absence or presence of telmisartan. The amounts of p-p38MAPK/p38MAPK (C) and pHSP27/HSP27 (D) were determined by immunoblot analyses. Representative blots are shown (upper panel). Results are expressed as the mean ± SD of a representative experiment ($n = 4$) for (A and B) and of four independent experiments ($n = 4$) for (C and D). NS: not significant, and P values, significantly different from cells incubated under the indicated conditions, according to a one-way ANOVA with Scheffe's (A, C and D) and Fisher's (B) post hoc comparison.

3. Results

3.1. TNF-α induced VEGF-C expression via the p38MAPK/HSP27 pathway

The treatment with TNF-α for 24 h increased VEGF-C mRNA levels in HPTECs in a dose-dependent manner (Fig. 1A). TNF-α (10 ng/mL) increased VEGF-C mRNA and protein levels by 2.8-fold and 3.6-fold, respectively (Fig. 1A and B). As shown in Supplementary Fig. 1 (Fig. S1), TNF-α appeared to increase the phosphorylation of p38MAPK, CREB, ERK1/2, and NFkB by as early as 5 min. Treatments with several specific inhibitors of the signaling pathways revealed that only SB203580, a specific inhibitor for p38MAPK, significantly decreased TNF-α-induced VEGF-C mRNA levels (Fig. 1C), whereas H89 and PD95089, inhibitors for mitogen-and stress-activated protein kinase 1 and MAPK/ERK kinase, respectively, or an NFkB inhibitor had no influence on this expression (Fig. 1D and Fig. S2A and B). SB203580 also significantly decreased TNF-α-induced VEGF-C protein levels (Fig. 1E). These results indicated that the activation of p38MAPK was responsible for the TNF-α-induced expression of VEGF-C.

HSP27 and CREB have been identified as main components of the p38MAPK signaling pathway; therefore, we investigated the time course of phosphorylation for these signaling components in detail. The phosphorylation of p38MAPK was maximal after 5–15 min (Fig. 2A), while that of HSP27 and CREB peaked after 15–30 min (Fig. 2B) after 15 min, respectively (Fig. S3). The inactivation of p38MAPK by SB203580 completely diminished the phosphorylation of HSP27 (Fig. 2C). Furthermore, siRNA-mediated HSP27 silencing caused an approximately 45% reduction in the TNF-α-stimulated expression of VEGF-C (Figs. S4, 2D), while CREB silencing had no effect on this expression (Fig. S5A and B). These

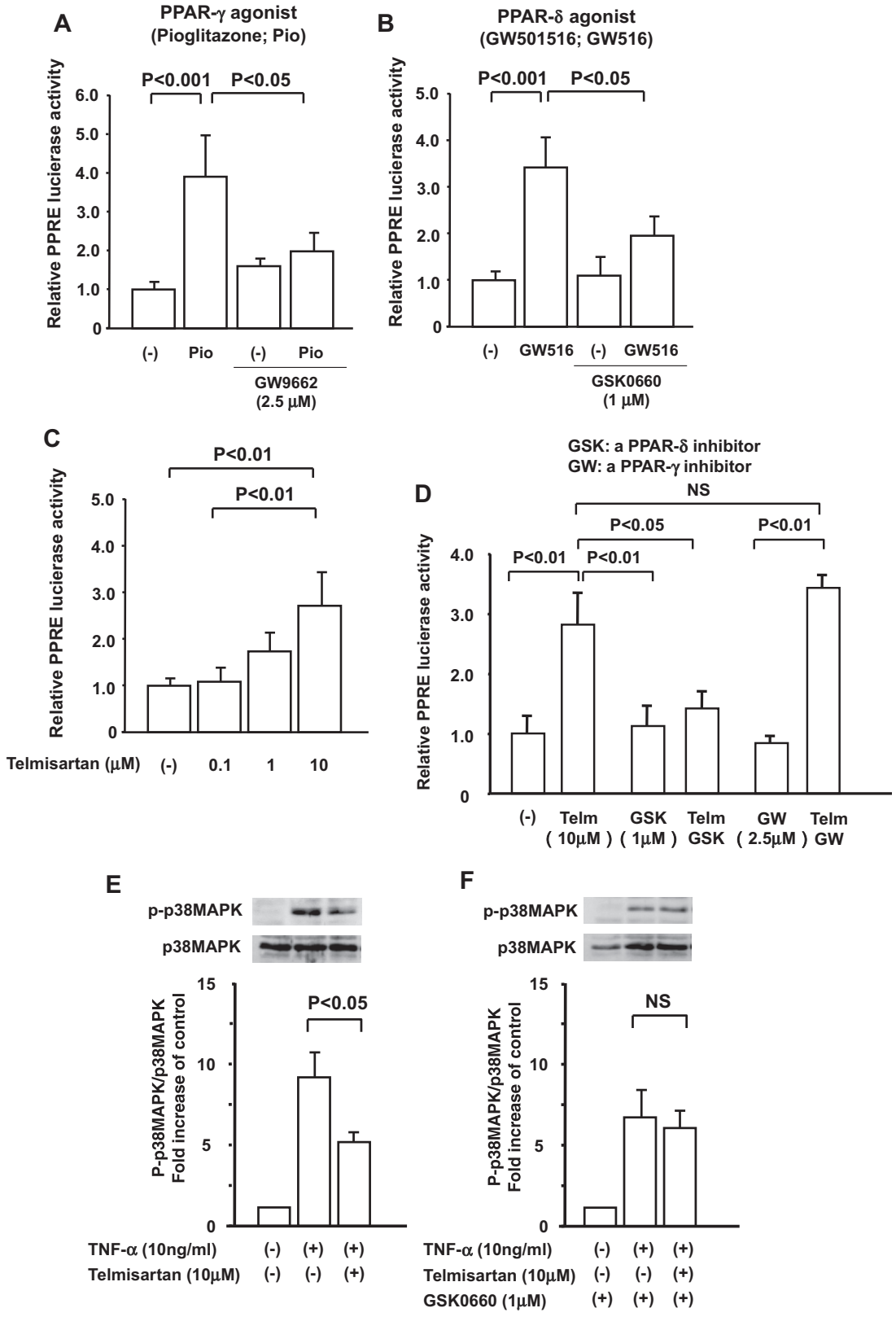

Fig. 4. Telmisartan activated the endogenous PPAR-δ protein and partially reduced the phosphorylation of p38MAPK in a PPAR-δ-dependent manner, causing reductions in the production of VEGF-C. (A and B) HPTECs were transfected with the PPRE-luciferase reporter construct, followed by a 24-h treatment with pioglitazone (A; 3 μM) or GW501516 (B; 3 μM) in the presence or absence of GW9662 or GSK0660, respectively. (C) HPTECs were transfected with the PPRE-luciferase reporter construct, followed by a 24-h treatment with the indicated concentrations of telmisartan. (D) HPTECs were transfected with the PPRE-luciferase reporter construct, followed by a 24-h treatment with or without telmisartan in the presence or absence of GW9662 (GW) or GSK0660 (GSK). (E and F) HPTECs were treated for 15 min with vehicle alone, TNF-α alone, or TNF-α plus telmisartan in the absence (E) or presence (F) of GSK0660. The amounts of p-p38MAPK/p38MAPK were determined by an immunoblot analysis. Representative blots are shown (upper panel). Results are expressed as the mean ± SD of a representative experiment (n = 3–6) for (A–D) and of three independent experiments for (E and F). *P* values, significantly different from cells incubated under the indicated conditions according to a one way ANOVA with Scheffe's (A and C–F) and Fisher's (B) post hoc comparison.

results suggested that TNF-α increased the expression of VEGF-C through the p38MAPK/HSP27 pathway.

3.2. Telmisartan decreased TNF-α-induced VEGF-C expression by suppressing p38MAPK/HSP27

We examined the effects of telmisartan, a representative ARB used to treat chronic inflammatory kidney diseases, on the TNF-α-induced production of VEGF-C in HPTECs. Telmisartan (10 μM) reduced TNF-α-stimulated VEGF-C expression by 25% at the mRNA level (Fig. 3A) and by 40% at the protein level (Fig. 3B). Moreover, telmisartan significantly diminished not only the phosphorylation of p38MAPK (Fig. 3C), but also its downstream event, the phosphorylation of HSP27 (Fig. 3D).

3.3. Telmisartan partially decreased TNF-α-induced VEGF-C expression in a PPAR-δ-dependent manner

We previously reported that telmisartan served as a PPAR-δ agonist [24]. Therefore, we investigated whether this was also the case in HPTECs. In HPTECs producing PPAR-α and -δ proteins (Fig. S6), the expression of PPRE-luciferase was enhanced by pioglitazone, an authentic PPAR-γ agonist, but was significantly inhibited by GW9662, a specific antagonist of PPAR-γ (Fig. 4A). GW501516, an authentic PPAR-δ agonist, also increased PPRE-luciferase activity, whereas this was significantly inhibited by GSK0660, a specific antagonist of PPAR-δ (Fig. 4B). Telmisartan dose-dependently increased the transcriptional activity of PPAR (Fig. 4C) and exhibited specific activity for PPAR-δ, but not PPAR-γ (Fig. 4D). GSK0660 significantly weakened the VEGF-C-reducing effects of telmisartan in HPTECs stimulated with TNF-α (Fig. S7). The telmisartan-induced percentage reductions in VEGF-C mRNA were significantly lower with the GSK0660 treatment than with no treatment (Fig. S7). GSK0660 also diminished the inhibitory effects of telmisartan on TNF-α-induced p38MAPK phosphorylation (Fig. 4E and F). Angiotensin II had a negligible influence on the expression of VEGF-C (Fig. S8) while eprosartan, a non-PPAR activating ARB, had no inhibitory effects on the TNF-α induced expression of VEGF-C (Fig. S9). These results indicated that telmisartan partially decreased the TNF-α-induced expression of VEGF-C by reducing the phosphorylation of p38MAPK/HSP27 in a PPAR-δ-dependent manner.

4. Discussion

In the present study, we demonstrated that TNF-α increased the expression of VEGF-C by activating the p38MAPK/HSP27 pathway, but not the ERK, CREB, or NK-kB pathways in HPTECs. We also found that telmisartan, a unique ARB with potentially agonistic effects on PPARs, served as a PPAR-δ, but not -γ activator, and decreased the TNF-α-induced expression of VEGF-C by partially inhibiting the p38MAPK/HSP27 pathway in a PPAR-δ-dependent manner.

Inflammatory cytokines such as IL-1β, TNF-α, heregulin-β1, or receptor activator of NF-κB ligand (RANKL) may modulate inflammation or promote tumor spread through VEGF-C-enhanced lymphangiogenesis depending on individual pathological conditions [9–11,26]. VEGF-C is also an inducer of lymphangiogenesis in the tubule-interstitial lesions of progressive renal diseases in which inflammation with macrophage infiltration and ensuing fibrosis can occur [6–8]. Several previous studies reported that p38MAPK/NF-kB and RANKL/NF-kB pathways were involved in the induction of VEGF-C by inflammatory cytokines in breast cancer cells [11] and osteoclasts, respectively [10]. Although TGF-β1 and TNF-α were shown to increase the expression of VEGF-C in renal tubular cells [6–8], the molecular mechanism responsible were unclear. The present study revealed for the first time that the p38MAPK/HSP27 pathway was involved in the TNF-α-induced expression of VEGF-C in HPTECs. The result was strongly supported by the findings of a previous study in which the knockdown of HSP27 reduced the secretion of VEGF-C in an angiogenic breast cancer cell line [27].

Another major finding of our study was the unique pleiotropic effects of telmisartan on TNF-α-induced inflammation in HPTECs. Namely, telmisartan activated the endogenous PPAR-δ protein and then decreased the TNF-α-induced expression of VEGF-C by partially inhibiting the p38MAPK/HSP27 pathway in a manner that depended on the action of PPAR-δ. Many previous studies identified telmisartan as a partial PPAR-γ activator [16–18], whereas telmisartan was recently shown to enhance PPAR-δ, but not -γ activity [19,20,24]. The telmisartan-induced activation of PPAR-γ reportedly exerted anti-inflammatory effects at the cellular and *in vivo* levels [16], a wide range of which were verified by the specific PPAR-γ inhibitor, GW9662. We previously reported that telmisartan not only enhanced PPAR-δ functions, but also induced anti-fibrotic effects in human mesangial cells, supporting the findings by Zhu and coworkers in which telmisartan was identified as a PPAR-δ activator using PPAR-δ-deficient mice. These findings were further confirmed by our current study using HPTECs. Based on previous findings confirming telmisartan as a PPAR-γ activator, we speculated that the preferences of telmisartan toward PPAR-δ and/or PPAR-γ may be cell-specific; however, the mechanisms responsible remain unclear. Regarding their effects on signaling pathways, the overexpression of PPAR-δ or a PPAR-δ agonist (GW0742) was shown to attenuate the phosphorylation of p38MAPK in rat mesangial cells, while a PPAR-δ inhibitor (GSK0660) increased it [28]. These are consistent with the effects of telmisartan as a PPAR-δ agonist, found in our study. Furthermore, GW501516, another PPAR-δ agonist, was reported to ameliorate tubulointerstitial inflammation in proteinuric kidney diseases of mice [29], in which VEGF-C expression may be up-regulated. In proximal tubular cells, GW501516 reduced TNF-α-stimulated expression of monocyte chemoattractant protein-1 (MCP-1) via inhibition of TGF-β activated kinase 1 (TAK-1)-NFkB pathway in a PPAR-δ-independent manner [29]. Likewise, telmisartan may also diminish MCP-1 expression.

In conclusion, we demonstrated that TNF-α increased the production of VEGF-C by activating the p38MAPK/HSP27 pathway in HPTECs, and this was partially inhibited by telmisartan in a PPAR-δ dependent manner. These results provide a novel insight into inflammation-associated lymphangiogenesis including tumor spread.

Acknowledgments

This study was supported by Grants-in-Aid for Scientific Research (c) (No. 24591193) and Grants-in-Aid for Scientific Research (b) (No. 24390216) from the Japan Society for the Promotion of Science.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.10.077.

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