*Full Paper***Troglitazone Inhibits Vascular Endothelial Growth Factor–Induced Angiogenic Signaling via Suppression of Reactive Oxygen Species Production and Extracellular Signal–Regulated Kinase Phosphorylation in Endothelial Cells**Byung Chul Park¹, Dinesh Thapa¹, Jong Suk Lee¹, Su-Young Park¹, and Jung-Ae Kim^{1,*}¹College of Pharmacy, Yeungnam University, Gyeongsan 712-749, Korea

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Abstract. Thiazolidinediones, peroxisome proliferators-activated receptor gamma (PPAR γ) ligands, have been recognized as a potential therapeutic agents for the treatment of pathological neovascularization. In the present study, we examined the molecular mechanism by which troglitazone (TROG), a PPAR γ agonist, exerts its inhibitory action in vascular endothelial growth factor (VEGF)-induced angiogenesis signaling. In an in vitro angiogenesis model using human umbilical vein endothelial cells, TROG (20 μ M) significantly suppressed VEGF-induced cell proliferation and invasion of the cells into the Matrigel basement membrane, which was not reversed by treatment with PPAR antagonists, GW9662 (10 μ M) and bisphenol A diglycidyl ether (10 μ M). TROG also blocked VEGF-induced reactive oxygen species (ROS) production and its downstream extracellular signal–regulated kinase (ERK) phosphorylation, and this inhibitory effect was not reversed by GW9662 (10 μ M). The antiangiogenic activity of TROG correlated with suppression of VEGF-induced matrix metalloproteinase (MMP)-2 and membrane type 1 (MT1)-MMP expression. In addition, the effects of TROG on VEGF-induced MMP-2 and MT1-MMP expression were comparable to those of the NADPH oxidase inhibitor diphenylene iodium (10 μ M) and ERK inhibitor PD98056 (10 μ M). Furthermore, in an in vivo angiogenesis system using a chick chorioallantoic membrane model, TROG dose-dependently inhibited VEGF-induced angiogenesis, which was similar to the inhibitory effect of *N*-acetylcysteine on VEGF-induced angiogenesis. The results suggest that the inhibitory effects of TROG on VEGF-induced angiogenesis were mediated through the suppression of VEGF-induced ROS production and ERK phosphorylation.

Keywords: angiogenesis, troglitazone, matrix metalloproteinase (MMP), reactive oxygen species (ROS), extracellular signal–regulated kinase (ERK)

Introduction

Angiogenesis, the growth of new capillaries from preexisting micro-vessels, plays an important role not only in physiological process such as embryonic development and wound healing, but also in the progression of various pathological conditions such as cancers, rheumatoid arthritis, and diabetic retinopathy (1, 2). Vascular endothelial growth factor (VEGF) is the most

well-known proangiogenic factor, and its action is primarily mediated through interaction with its receptor VEGFR2 (3). The activation of VEGFR2 is known to be associated with the activation of NADPH oxidase, which generates reactive oxygen species (ROS) and subsequent activation of the mitogen-activated protein kinase (MAPK) cascade during angiogenesis (4 – 6).

For migration and invasion of endothelial cells into the surrounding tissue during angiogenesis, proteases such as matrix metalloproteinases (MMPs) are necessary to degrade tissue barriers. MMP-2 and MMP-9 are the major MMP species for degrading type IV collagen, a major component of the basement membrane (7). They

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are abundantly expressed in cancer cells (8, 9) and vasculature (10, 11) and play an important role in not only the migration of inflammatory cells and tumor cells into the basement membrane, but also in the sprouting of endothelial cells by VEGF (12). MMP-2 is secreted as an inactive zymogen that can be activated at the cell membrane via the membrane-type MMP (MT1-MMP) (13). ROS is an important signal required for the activation and expression of pro-MMP-2 in both cancer cells (14) and vascular cells (15).

Peroxisome proliferators-activated receptor gamma (PPAR γ), one of the PPAR members of the nuclear hormone receptor superfamily, plays an important role in obesity, diabetes, inflammation, and tumorigenesis by regulating cellular proliferation and differentiation. An increasing line of evidence has shown that PPAR γ ligands also exert inhibitory effects on the cytokine-induced proliferation and migration of various types of cells (16–18). In leukemia cells and vascular smooth muscle cells, the anti-migratory action of troglitazone (TROG), one of the PPAR γ agonists, correlates with its regulatory effects on MMP expression (19, 20). However, in vascular endothelial cells, only TROG, but not pioglitazone and rosiglitazone, exerts a regulatory effect on MMP expression (21). These reports suggest that TROG, among the PPAR γ ligands, may serve as an anti-angiogenic agent by inhibiting proliferation, migration, and MMP expression in endothelial cells. However, the detailed signaling pathway of TROG involved in the anti-angiogenic action is not yet available.

In the present study, we investigated the effects and action mechanism of TROG on the VEGF-induced angiogenesis in relation to MMP activation and expression.

Materials and Methods

Materials

The basic form of VEGF was purchased from R&D Systems (Minneapolis, MN, USA). Endothelial growth medium (EGM)-2 bullet kit containing an endothelial cell basal medium (EBM)-2 and EGM-2 SingleQuots (hydrocortisone, hFGF, VEGF, R3-IGF-1, ascorbic acid, hEGF, heparin, gentamicin, and FBS) was purchased from Clonetics (San Diego, CA, USA). HEPES-buffered saline solution, trypsin/EDTA, and trypsin neutralizing solution (TNS) were purchased from Clonetics, Inc. (Walkersville, MD, USA). The polymer Matrigel was purchased from BD Biosciences (Bedford, MA, USA). The [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), 2% gelatin solution, *N*-acetyl-L-cysteine (NAC), TROG, diphenylene iodium (DPI), PD98059, cortisone acetate, and 2',7'-dichlorofluorescein diacetate

(DCF-DA) were purchased from Sigma (St. Louis, MO, USA).

Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (San Diego, CA, USA). HUVECs were grown to 80%–90% confluence in EBM-2 containing hydrocortisone, hFGF, VEGF, R3-IGF-1, ascorbic acid, hEGF, heparin, gentamicin, and 2% FBS equilibrated with 5% CO₂ at 37°C. HUVECs were serially passaged and maintained in EBM-2 in cell culture flasks coated with 0.2% gelatin (Sigma). Confluent cultures of HUVECs between the third and sixth passages were washed with HEPES-buffered saline solution and harvested using trypsin/EDTA and neutralization by TNS.

Cell proliferation assay

HUVECs plated at a density of 2×10^4 cells/well in 48-microwell plates were incubated for 24 h in EBM-2 containing only 1% FBS and cotreated with VEGF (20 ng/ml) and TROG for 48 h. After incubation, an MTT assay was carried out and the optical density was measured at 540 nm using a microplate reader (Versa MAX; Molecular Devices, Sunnyvale, CA, USA).

Determination of intracellular of ROS production

Intracellular ROS generation was measured using 2',7'-dichlorofluorescein diacetate (DCF-DA), a fluorescent dye (22). Confluent cells were pretreated with inhibitors and then treated with 20 ng/ml VEGF. After incubation for 5 min, the cells were loaded with 10 μ M DCF-DA for 5 min at 37°C and imaged by an inverted fluorescence microscopy (TE2000-U; Nikon, Tokyo).

Tube formation assay

The tube formation assays were performed on 48-well plates coated with 100 μ l of Matrigel basement membrane matrix per well and polymerized at 37°C for 30 min. HUVECs were suspended in 2% FBS and another supplement containing EBM-2 medium. The cells were plated on Matrigel at a density of 5×10^4 cells per well, and test compound was added to the culture medium. After 14 h, four fields were randomly selected from each culture and photographed with a CCD camera (TE2000-U, Nikon).

In vitro invasion assay

An in vitro invasion assay was performed using a 24-transwell unit (8- μ m pore size) with polycarbonate filters (Corning, Cambridge, MA, USA) by the previously described method (23). The upper and lower sides of the transwell filter were coated with 40 μ l of

Matrigel (0.5 mg/ml) (BD Biosciences) and type I collagen (0.5 mg/ml), respectively. The lower compartment was filled with EBM-2 containing EGM-2 SingleQuots and 1% FBS. The cells were placed in the upper part of the transwell plate and incubated with VEGF (20 ng/ml) for 24 h at 37°C. TROG was co-administered with VEGF, and pretreatment with the other inhibitors was performed 30 min before VEGF treatment. The cells that invaded the lower surface of the membrane were fixed with methanol and stained with hematoxylin and eosin. We determined invasive phenotypes by counting the cells that migrated to the lower side of the filter using $\times 200$ magnification. Three fields were counted for each filter.

Zymography

The enzymatic activities of MMP-2 and MMP-9 were assayed by gelatin zymography (24) in the absence of serum. Supernatants from TROG with or without VEGF cultures were electrophoresed using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) containing gelatin. The gel was washed twice with washing buffer [50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 2.5% Triton X-100], followed by a brief rinsing in washing buffer without Triton X-100, and then incubated with incubation buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM CaCl₂, 0.02% NaN₃] at 37°C. After incubation, the gel was stained with 0.25% Coomassie Brilliant Blue R250 (Sigma) and then destained. A clear zone of gelatin digestion represented MMP activity.

MMP-2 ELISA

An enzyme-linked immunosorbent assay (ELISA) kit (Oncogene Research Products, Boston, MA, USA) was used to measure the extracellular protein levels of MMP-2 according to the manufacturer's protocol. Three independent experiments in duplicate were performed.

Quantitative real-time polymerase chain reaction (PCR)

Cells were collected and total RNA was extracted with a spin column using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Isolated mRNA was reverse-transcribed by using the Reverse Transcription kit (Qiagen). The gene expression levels were analyzed by a Quantitative Real-Time PCR system (Rotor-Gene 6000; Corbett, San Francisco, CA, USA). Real-time PCR was performed with the SYBR Green PCR kit (Qiagen). The following primers were used for PCR analysis: MMP-2 stock primer (Qiagen Cat. No. QT00088396) and MT1-MMP stock primer (Qiagen Cat. No. QT00001533). The reaction mixture consisted of 2 μ l of cDNA template,

10 μ l of SYBR Green PCR master mix, 5 pM of primers, and RNase free water in a total volume of 20 μ l. The cDNA was denatured at 95°C for 15 min followed by 55 cycles of PCR (95°C for 5 s, 55°C for 10 s, 72°C for 20 s). The mRNA levels of all genes were normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) stock primer (Qiagen Cat. No. QT00079247) as an internal control.

Western blotting

After HUVECs were treated with the test drugs, the cells were immediately washed with ice-cold PBS and scraped with a rubber policeman in ice-cold homogenization buffer [20 mM HEPES (pH 7.4), 1 mM EDTA, 20 μ g/ml leupeptin, 10 μ g/ml aprotinin, 2 μ g/ml pepstatin, 1 mM PMSF, 1 mM DTT]. Total proteins were extracted using a lysis buffer [20 mM HEPES (pH 7.5), 20 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 mM Na₃VO₃, and 1X Protease Inhibitor Cocktail], and samples (each containing 30 μ g of protein) were separated by SDS-PAGE. The resolved proteins were electrophoretically transferred onto a nitrocellulose membrane from the gel. The nitrocellulose blots were blocked for 1 h at 37°C using 5% skim milk in TBS-T buffer (TBS buffer containing 0.1% Tween-20). After 3 washes with TBS-T, the blots were incubated with primary antibodies, p-ERK (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and ERK (Cell Signaling, Beverly, MA, USA) in 5% skim milk in TBS overnight at 4°C on a shaker. For the detection of phosphorylated proteins, bovine serum albumin was used to block nonspecific binding. After 3 washes with TBS-T, the blots were incubated with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology) for 1 h at room temperature. Then the blots were washed again and visualized with enhanced chemiluminescence (ECL) detection. The abundance of each protein was detected and normalized to that of actin. This experiment was repeated three times.

Chick chorioallantoic membrane (CAM) assay

The 10-day-old embryos were purchased from Baek-ja farm (Cheongsong, Korea) and were incubated at 37°C with 55% relative humidity. A small hole was punctured in the shell concealing the air sac using a hypodermic needle. A second hole was punctured in the shell on the broadside of the egg directly over the avascular portion of the embryonic membrane, as observed during candling. A false air sac was created beneath the second hole by the application of negative pressure to the first hole, which caused the CAM to separate from the shell. A window, approximately 1.0 cm², was cut in the shell

Fig. 1. Effects of TROG on VEGF-induced HUVEC proliferation. A and B: The serum-starved HUVECs (1×10^4 cells/well) were stimulated with 20 ng/ml of VEGF in the presence or absence of TROG for 48 h, and then the number of viable cells was measured using the MTT method. The cells were pretreated with GW9662 for 30 min prior to co-treatment with VEGF and TROG. C: HUVECs were treated for 48 h with or without each concentration of GW9662 or BADGE, and cell viability was measured using the MTT assay. Data are each the mean $\pm$ S.E.M. from three independent experiments. * $P < 0.05$, compared to the vehicle-treated control; # $P < 0.05$, compared to the VEGF-treated group.

over the dropped CAM with the use of a small crafts grinding wheel (Dremel, Racine, WI, USA). Sterile filter disks (Whatman No.1 filter paper; Maidstone, UK) were soaked in 3 mg/ml cortisone acetate in a solution of 90% ethanol and water and subsequently air-dried under sterile conditions. VEGF was used to grow vessels on the CAMs of 10-day-old chick embryos. Sterile filter disks, absorbed with VEGF (20 ng/ml) dissolved in PBS containing 0.1% BSA, were placed on the growing CAMs. Then, test compounds or vehicle was added directly to the CAMs topically. CAM tissue directly beneath the VEGF-saturated filter disks was resected from embryos treated 72 h previously with compound or vehicle. CAM disks were harvested for light microscopy (Leica, Germany). The number of vessel branch points contained in a circular region equal to the area of filter disk was counted for each section.

Statistics

The data are each expressed as the mean $\pm$ S.E.M. and were analyzed by one-way analysis of variance (ANOVA) and the Student-Newman-Keul's test for individual comparisons. P values of < 0.05 were considered statistically significant.

Results

TROG inhibits VEGF-induced HUVEC proliferation via suppression of ROS and ERK phosphorylation

Since the angiogenic process includes the proliferation, migration, and tube formation of endothelial cells (ECs), we examined the effects of TROG on each of the angiogenic processes in vitro using HUVECs. To examine the effect of TROG on the VEGF-induced endothelial cell proliferation, an MTT assay was performed. Treatment of the serum-starved HUVECs with VEGF (20 ng/ml) for 48 h increased proliferation of

Fig. 2. VEGF-induced ROS production in HUVECs was inhibited by TROG and DPI, but not by PD98059. A: Serum-starved HUVECs were stimulated with VEGF (20 ng/ml) for 5 min in the presence or absence of drugs and then incubated with DCF-DA (5 μ M) for an additional 5 min. In the case of DPI and PD98059, the drug was co-administered with VEGF; and for GW9662 treatment, cells were pretreated with the drug for 30 min prior to VEGF treatment. The cellular ROS level was captured under a fluorescence microscope (TE2000-U; Nikon, Tokyo) at $\times 200$ magnification, and the images were imported and analyzed by using the Image-Inside program. B: The cell proliferation was measured as described in Fig. 1A. Data are each the mean $\pm$ S.E.M. from three independent experiments. * $P < 0.05$, compared to the vehicle-treated control; # $P < 0.05$, compared to the VEGF-treated group.

the cells, and this VEGF-induced proliferation was significantly suppressed by TROG (20 μ M) (Fig. 1: A and B). In order to determine whether the effect of TROG was PPAR γ -dependent, the cells were treated with GW9662 (10 μ M) and bisphenol A diglycidyl ether (BADGE) (1, 5, 10 μ M), PPAR antagonists, in combination with TROG. In fact, PPAR antagonists failed to reverse the anti-proliferative effect of TROG (Fig. 1: A and B). Rather, the addition of GW9662 and BADGE enhanced the inhibitory effects of TROG. Of particular note was that the treatment of BADGE alone

significantly reduced the cell viability (Fig. 1C).

Since the VEGF-induced proliferation is mediated through the generation of ROS by NADPH oxidase (5), which in turn, activates ERK, we also tested whether the anti-proliferative effect of TROG was due to the suppression of ROS and ERK (25). TROG (20 μ M) significantly inhibited the VEGF-induced ROS generation (Fig. 2A), which was comparable to the effects of treatment with DPI (10 μ M), an NADPH oxidase inhibitor, on VEGF-induced ROS generation (Fig. 2A) as well as cell proliferation (Fig. 2B). Such an inhibitory

Fig. 3. TROG and DPI inhibits VEGF-induced ERK phosphorylation. A: HUVECs were treated with VEGF in the absence or presence of TROG for the indicated time. B: The cells were co-treated with VEGF and PD98059 or DPI for 5 min. Protein lysates were extracted and analyzed for the expression level of phospho-ERK and ERK by immunoblotting. In the bar graph, the relative expression of phosphorylated ERK normalized to ERK level is expressed as the mean $\pm$ S.E.M. * P <0.05, compared to the vehicle-treated control; # P <0.05, compared to the VEGF-treated group.

effect of TROG was not reversed by co-treatment with GW9662 (10 μ M). Rather, GW9662 itself suppressed the VEGF-induced ROS generation. In the case of the treatment with PD98059 (10 μ M), an ERK1/2 inhibitor, it did not suppress the VEGF-induced ROS, but significantly suppressed the VEGF-induced endothelial cell proliferation (Fig. 2B). Moreover, as shown in Fig. 3A, treatment of HUVECs with VEGF significantly induced phosphorylation of ERK, most strongly at 5 min and then decreased shortly thereafter. This VEGF-induced ERK phosphorylation was suppressed by treatment with TROG (20 μ M). In a parallel experiment, DPI (10 μ M) also suppressed the VEGF-induced ERK phosphorylation in a similar manner to TROG (Fig. 3B).

TROG suppresses tube formation and invasion of human endothelial cells by VEGF

When HUVECs were placed on Matrigel in the presence of VEGF (20 ng/ml), the cells rapidly aligned with one another and formed tube-like structures within 12 h. Treatment of the cells with TROG (10 and 20 μ M) concentration-dependently prevented the VEGF-stimulated tube formation of HUVECs (Fig. 4A). Similarly, TROG significantly suppressed VEGF-induced invasion of the cells in the Matrigel invasion assay (Fig. 4B). In

addition, the effect of TROG (20 μ M) was comparable to those of DPI (10 μ M) and PD98059 (10 μ M). On the other hand, the inhibitory action of TROG on VEGF-induced invasion of HUVECs was not reversed by co-treatment with GW9662 (10 μ M). The treatment with a drug alone (TROG, GW, DPI, and PD) in the absence of VEGF did not alter the invasive behavior of the cells (Fig. 4C).

TROG inhibits VEGF-induced MMP-2 and MT1-MMP expressions in HUVECs

For the migration and invasion of endothelial cells into the surrounding tissue during angiogenesis, the cells require proteases such as MMPs to degrade tissue barriers presented by basement membranes and the interstitial matrix. We examined the effect of TROG on VEGF-induced MMP-2 and MT1-MMP level in HUVECs.

In a real-time PCR assay, treatment of the cells with TROG (10 and 20 μ M) significantly suppressed not only the basal expression of MMP-2 and MT1-MMP but also the VEGF-induced MMP-2 (Fig. 5A) and MT1-MMP (Fig. 5B) mRNA expression in a concentration-dependent manner. This inhibitory effect of TROG was stronger than the effect produced by treatment with

Fig. 4. Inhibitory effect of TROG on VEGF-induced tube formation and invasion of HUVECs. **A:** HUVECs were plated on a well coated with 40 μ l of Matrigel basement membrane matrix. After they were treated with VEGF and TROG for 12 h, the cells were photographed using a digital camera attached to a phase contrast microscope at $\times 100$ magnification. **B and C:** The lower and upper parts of the transwell insert were coated with collagen and Matrigel, respectively. HUVECs were treated with TROG, DPI, PD98059, or GW9662 in the presence (B) or absence (C) of VEGF. After 24 h, the cells on the bottom side of the filter were fixed, stained, and captured under a microscope. The bar graphs represent the mean number of cells invaded per field $\pm$ S.E.M. of three independent experiments. * $P < 0.05$, compared to the untreated control; # $P < 0.05$, compared to the VEGF-treated group.

DPI (10 μ M) or PD98059 (10 μ M) (Fig. 5C and 5D). In a similar manner, TROG inhibited VEGF-induced MMP-2 protein secretion measured by ELISA (Fig. 5E) and MMP-2 activity revealed in the zymogram assay (Fig. 5F). In contrast to MMP-2, MMP-9 activity was very weak, and not affected by VEGF and TROG.

TROG inhibits angiogenesis in vivo

To verify the anti-angiogenic activity of TROG in vivo, we performed a CAM assay. TROG alone had no effect on neovascularization on chick embryo CAM (Fig. 6). However, VEGF (20 ng/ml)-stimulated blood vessel formation was significantly suppressed by treatment of CAM with TROG (0.05, 0.1, and 0.2 μ g/CAM) in a dose-dependent manner. The inhibitory effect of

Fig. 5. TROG inhibits VEGF-induced MMP-2 and MT1-MMP expression in HUVECs. A and B: Quantitative real-time PCR for MMP-2 (A) and MT1-MMP (B) mRNA expression was performed using the SYBR Green PCR kit (Qiagen). The data represent the mean of three different sets of experiments conducted in triplicate. The black and gray colors in the bar graphs represent the HUVECs treated with TROG in the absence or presence of VEGF, respectively. C and D: HUVECs were treated with DPI or PD98059 for 24 h in the presence of VEGF (20 ng/ml). Quantitative real-time PCR for MMP-2 (C) and MT1-MMP (D) mRNA expression was performed as described in A and B. E and F: HUVECs were co-treated with VEGF and TROG for 24 h. The secreted amount of MMP-2 (E) in the conditioned media was measured by using ELISA and normalized by the total number of viable cells determined by an MTT method. The conditioned media were also analyzed for gelatinolytic activity of MMP-2 by zymography (F). The black and gray bar graphs under the zymogram represent the relative band intensity of pro-MMP-2 and active MMP-2, respectively. * $P < 0.05$, compared to the vehicle-treated control group; # $P < 0.05$, compared to the VEGF-treated group.

Fig. 6. TROG inhibits VEGF-induced angiogenesis in CAM assay. VEGF (20 ng/ml) or vehicle (0.1% bovine serum albumin in PBS) was placed on the CAM of a 10-day-old chick embryo. TROG and NAC were introduced 30 min later on top of the CAMs by means of the filter disks. After 3 days of incubation, digital images (magnification: $\times 50$) of CAM sections exposed to filters were captured as representative images and imported into the image analysis program to quantify the number of new vessel branches formed. V represents VEGF. The bar graphs each represent the mean $\pm$ S.E.M. of at least six chick embryos. * $P < 0.05$, compared to the vehicle-treated control group; # $P < 0.05$, compared to the VEGF-treated group.

TROG was more obvious than that of NAC, an anti-oxidant (Fig. 6).

Discussion

Accumulating evidence has contributed toward elucidating the versatile role of PPAR γ in modulating diverse biological functions such as lipid biosynthesis and glucose metabolism, as well as cell fate determination (1–3, 26, 27). Furthermore, since various PPAR γ ligands inhibited growth and migration of vascular endothelial cells, smooth muscle cells, monocytes, and certain tumor cells (28–30), PPAR γ has been recognized as a potential therapeutic target for the treatment of pathological neovascularization (31, 32). There are several lines of in vitro and in vivo evidence to support the postulation that a PPAR γ ligand can also induce tumor angiogenesis (33–36) through an indirect action on endothelial cells by inducing the expression of the proangiogenic factor VEGF or PPAR γ coactivator (PGC)-1 α in cancer cells. However, the apoptosis-inducing ability of PPAR γ activators in malignant transformed cells (37–39) may surpass the reality of the pro-angiogenic factor expression in tumor tissues. Furthermore, recent investigations supported the importance of endothelial cells among many cell types

in relation to PPAR γ -mediated anti-angiogenic therapy because endothelial cells express functionally active PPAR γ (30, 40, 41). 15d-PGJ₂, an endogenous PPAR γ ligand, and thiazolidinediones, the best known exogenous PPAR γ ligands, inhibit endothelial proliferation and differentiation into tube-like structures (31, 42). Rosiglitazone directly suppressed the growth of a variety of tumors and metastatic invasion by inhibiting angiogenesis (41). In addition, TROG inhibited VEGF-induced cell proliferation, migration, and tube formation of bovine choroidal endothelial cells (31). Similarly, the present study clearly demonstrated that TROG inhibited VEGF-induced angiogenesis in both the in vivo chick CAM assay and in vitro human umbilical endothelial cell proliferation, migration and tube formation analysis.

Increasing evidence suggests that many angiogenic responses of endothelial cells are dependent on ROS generated by endothelial NADPH oxidase upon stimulation by angiogenic factors (5, 6, 43, 44). The effects of PPAR γ agonists on cellular ROS level vary depending on the type of stimuli administered to the cell. Treatment of glioma or osteoblastic cells with ciglitazone or TROG alone increases the ROS level (45, 46). However, when the cells are co-treated with other agents such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and octanoate,

all of which stimulate cellular proliferation or lipogenesis, PPAR γ agonists suppress the agent-induced ROS generation (47, 48). Furthermore, in leukocytes isolated from TROG-treated obese patients, TROG was found to suppress the ROS generation (49, 50). The present study clearly showed that TROG inhibited VEGF-induced NADPH oxidase-mediated ROS production (Fig. 2A). The results obtained with endothelial cells correspond to reports by Jung et al. (51) showing that TROG suppressed the tumor necrosis factor (TNF)- α -stimulated NADPH oxidase 4 (NOX4)-dependent ROS generation. Moreover, our study also showed that this inhibitory effect of TROG was not suppressed by GW9662. However, the PPAR γ antagonist GW9662 alone blocked VEGF-induced ROS production, suggesting that ligand binding of PPAR γ may possibly induce direct inhibition of the VEGF-induced activation of NADPH oxidase.

Similar to the VEGF-induced HUVEC proliferation, ROS are reported to be involved in the activation and expression of pro-MMP-2 in vascular cells (15). Our results showed that the inhibitory effect of TROG on the VEGF-induced MMP-2 and MT1-MMP expression was similar to that of DPI treatment, an NADPH oxidase inhibitor. In addition, our results showed that TROG suppressed the VEGF-induced ERK phosphorylation and that MMP-2 and MT1-MMP expression was also inhibited by PD98059. Furthermore, PD98059 did not suppress the VEGF-induced ROS production, and DPI inhibited ERK phosphorylation, indicating that ROS are the upstream signal for the activation of ERK. These results suggest that the inhibitory effect of TROG on VEGF-induced MMP expression is mediated through the suppression of NADPH oxidase-generated ROS and ERK signaling.

TROG alone significantly suppressed the expression of MMP-2 and MT1-MMP in HUVECs. Nevertheless, TROG did not inhibit in vitro tube formation (Fig. 4A) and Matrigel invasion (Fig. 4C) of HUVECs and in vivo neovascularization as determined by the CAM assay (Fig. 6). These results suggest that down regulation of MMP-2 and MT1-MMP mRNA level is not enough for TROG action in the inhibition of angiogenesis, and other factors required for blood vessel formation may not be inhibited by TROG. One possible factor is tissue inhibitors of metalloproteinase (TIMP)-2. In the activation of MMP-2, TIMP-2 plays a dual role (52). Low levels of TIMP-2 are related to MT1-MMP-mediated activation of MMP-2, but high TIMP-2 levels directly inhibit MT1-MMP-mediated MMP-2 activation. In addition, MMP-1 and MMP-10, which are known to be involved in regression of human capillary tubular structures (53), may be other possible factors. Future

studies about the effect of TROG on these molecules are still needed.

The present study also showed that the concentration of TROG inducing anti-angiogenic activity was higher than that for receptor activation. These results are comparable to the previous report showing that high concentrations of rosiglitazone, which are greater than the concentration required for receptor activation, inhibit HUVEC proliferation (54). In addition, the PPAR γ antagonists BADGE and GW9662 did not block the inhibitory action of TROG in the VEGF-induced angiogenic effect. Since TROG is a PPAR γ -specific agonist as well as a ligand for human pregnan X receptor (PXR), we can not exclude the possibility that GW9662 may not be enough to block activation of PPAR γ , PXR, or the interaction between PPAR γ and PXR. Therefore, it can not be concluded that the anti-angiogenic action of TROG was mediated through a PPAR γ -independent pathway.

In conclusion, the anti-angiogenic activity of TROG is mediated through suppression of VEGF-induced ROS production, which leads to ERK phosphorylation.

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