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ABSTRACT

To investigate whether telmisartan (TMS), a unique angiotensin II type 1 receptor (AT1R) antagonist with selective peroxisome proliferator-activated receptor (PPAR) γ-modulating activity, inhibits plasminogen activator inhibitor 1 (PAI-1) and/or stimulates tissue-type plasminogen activator (t-PA) mRNA expression in vascular endothelial cells (VECs), we cultured VECs obtained from the thoracic aorta of Long Evans Tokushima Otsuka rats by the explant method. mRNA expression was measured using comparative reverse transcription polymerase chain reaction. The binding activity of PPARγ to its coactivator (CREB-binding protein) was determined by enzyme-linked immunosorbent assay. The synthetic PPARγ agonists troglitazone (TRO) and TMS activated PPARγ with EC_{50} values of 0.95 and 161 μM, respectively, under cell-free conditions. PPARγ mRNA was constitutively expressed in cultured VECs, unlike PPARα and PPARβ. PAI-1 mRNA expression was significantly downregulated by TMS, and this downregulation was not abolished by the presence of the PPARγ antagonist GW9662. The expression of t-PA mRNA was not altered by TMS treatment. TRO had no effect on PAI-1 and t-PA mRNA expressions. PAI-1 mRNA expression was significantly decreased by GW9662. In conclusion, TMS inhibited PAI-1 mRNA expression independently of PPARγ activation. Intrinsic PPARγ agonists, but not extrinsic PPARγ agonists, may be involved in basal mRNA expression of PAI-1 in VECs. *Ryukyu Med. J., 37 (1~4) 41~50, 2018*

Key words: telmisartan, peroxisome proliferator-activated receptor γ, angiotensin II type 1 receptor antagonist, vascular endothelial cells, plasminogen activator inhibitor 1

INTRODUCTION

Angiotensin II (Ang-II) is a powerful vasoconstrictor that increases intracellular Ca^{2+} by stimulating Ca^{2+} channels in vascular smooth muscle cells[1, 2)]. Telmisartan (TMS) exerts antihypertensive effects by blocking Ang-II type 1 receptor (AT1R) blocker (ARB) action[3)]. Interestingly, TMS does not have a biphenyl tetrazole moiety, which is found in all other ARBs[4)]. In

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addition, TMS has an activating effect on peroxisome proliferator-activated receptor (PPAR) γ in the CV-1 monkey kidney cell line, in contrast to other ARBs[5]. The PPARγ-activating effect of TMS might be associated with the lack of a biphenyl tetrazole moiety, although this remains to be clarified.

PPARγ is a member of the ligand-dependent nuclear hormonal receptor transcription superfamily, is highly expressed in adipocytes[6], and is also expressed in vascular endothelial cells (VECs). Previous studies showed that an intrinsic PPARγ agonist, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), downregulated endothelin-1 and upregulated plasminogen activator inhibitor 1 (PAI-1) in VECs[6-8]. In addition, expression of PAI-1 is significantly increased during 3T3-L1 adipocyte differentiation, and the PAI-1 expression positively correlates with the gene expression of PPARγ [9, 10]. A peroxisome proliferator-responsive element (PPRE)-like cis-element is present in the mouse *PAI-1* gene promoter[9] and treatment of human VECs with any of the intrinsic PPARγ agonists 15d-PGJ_2, 9-hydroxy-(S)-10,12-octadecadienoic acid, and 13(S)-10,12-octadecadienoic acid augments PAI-1 mRNA and protein expression[8]. Therefore, we hypothesized that TMS upregulates *PAI-1* gene expression through activation of PPARγ in VECs and promotes thrombogenesis as an adverse effect. In the present study, we investigated the effect of TMS on the expression of fibrinolytic factors, particularly PAI-1, in VECs. We also tested the involvement of PPARγ in the regulation of PAI-1 mRNA expression in VECs using troglitazone (TRO), a PPARγ agonist, and GW9662, a PPARγ antagonist.

MATERIALS AND METHODS

Animals and animal care

All animals were cared for according to the Standards Relating to the Care and Management of Experimental Animals (Notification No. 6; March 27, 1980; Prime Minister's Office, Tokyo, Japan) and the guide for animal experiments issued by the University of the Ryukyus. All animal studies were reviewed and approved by the Animal Care Committee at the University of the Ryukyus. Long Evans Tokushima Otsuka (LETO) rats were kindly provided by the Otsuka Pharmaceutical Tokushima Research Institute (Tokushima, Japan).

Drugs

TMS was kindly donated by Nippon Boehringer Ingelheim Co., Ltd. (Tokyo, Japan). Troglitazone (TRO), a PPARγ agonist, and GW9662, a PPARγ antagonist, were purchased from Sigma-Aldrich Japan Co., Ltd. (Tokyo, Japan). All drugs were dissolved in dimethyl sulfoxide (DMSO) and were modified by the addition of dilution buffer for PPARγ activity measurement and culture medium for the treatment of VECs.

Cell culture

Collagen-coated cultured dishes were sterilized by exposure to ultraviolet light overnight. LETO rats (56-64 weeks old) were administered intraperitoneal injections of pentobarbital sodium (75mg/kg body weight), and their thoracic aorta (length, approximately 3cm) was excised. The aorta was gently cleaned to remove periadventitial fat and connective tissue and was then cut into flat segments (approximately 5×5mm). The segments were plated on the dishes with the luminal side facing downward (4-6 segments per dish). The segments were cultured for 3 days under 5% CO_2 at 37℃ in Ham's F12-K medium (Sigma Chemical Co., St Louis, MO) containing 10% fetal bovine serum (FBS; Biosource International, Camarillo, CA) plus penicillin and streptomycin. The segments were removed on the third day after plating, and the cells attached to the bottom of the culture dishes were allowed to grow for several days until confluence. Any vascular smooth muscle cells and fibroblasts were carefully removed under microscopic observation by scraping with an autoclaved micropipette tip capped with a silicone tube.

Confluent VECs were treated with 0.5% trypsin-EDTA (5.3mmol/L) solution (Life Technologies Japan Ltd., Tokyo, Japan) and then passaged in 24-well culture dishes (Corning Co., Corning, NY) coated with type I collagen. Cultures of confluent VECs from the third to the sixth passages were used for the experiments. The VECs had a "cobblestone" appearance, where cells are in contact with neighboring cells (Fig. 1A).

PPARγ activity measurement

PPARγ activity was measured using EnBio RCAS for PPAR (EnBioTec Laboratories Co., Ltd., Tokyo, Japan). Under cell-free conditions, TMS, TRO, and GW9662 were incubated in the presence of PPARγ and then allowed to interact with an

immobilized coactivator, CREB-binding protein (CBP), in a 96-well microtiter plate. The amount of PPARγ bound to CBP was detected using horseradish peroxidase-conjugated anti-PPARγ antibody with the substrate 3,3′ ,5,5′ -tetramethylbenzidine.

Comparative RT-PCR

To measure mRNA expression, we performed comparative reverse transcription polymerase chain reaction (RT-PCR) as previously reported[11]. Briefly, total RNA was extracted from approximately 1×10^5 cultured cells by using the PURESCRIPT® RNA Isolation Kit (Gentra System, Minneapolis, MN). After reverse transcription with Moloney murine leukemia virus reverse transcriptase (8unit/mL) with an oligo $(dT)_{15}$ primer for 2 h at 37℃, the first-strand cDNA templates were amplified by 0.2 units of Taq DNA polymerase (Promega, Madison, WI) in 20μL of 10mmol/L Tris buffer (pH 9.0) containing 50mmol/L KCl, 0.2mmol/L of dNTPs, 2.5mmol/L $MgCl_2$, and 1 mmol/L of each primer. The primers used for targeting rat cDNAs of PPARα, PPARβ, PPARγ, PAI-1, t-PA, and glyceraldehyde-3-phosphate dehydrogenase are shown in Table 1, as are the comparative annealing temperatures and the number of polymerase chain reaction (PCR) cycles. The PCR products were separated on a 1.7% agarose gel containing ethidium bromide with a half concentration of Tris-borate-EDTA electrophoresis buffer (pH 7.5). The fluorescent intensities of amplified cDNA products were estimated by NIH ImageJ® (a public domain image processing and analysis program developed at the National Institute of Mental Health).

Cell proliferation assay

Cell proliferation of cultured VECs was examined by the Cell Proliferation Reagent WST-1 (Roche Diagnostics GmbH, Mannheim, Germany). The cultured VECs were trypsinized, and 3.0×10^3 cells in 100μl of 10% FBS-Ham's F12-K medium were plated in each well of a 96-well microplate (Corning

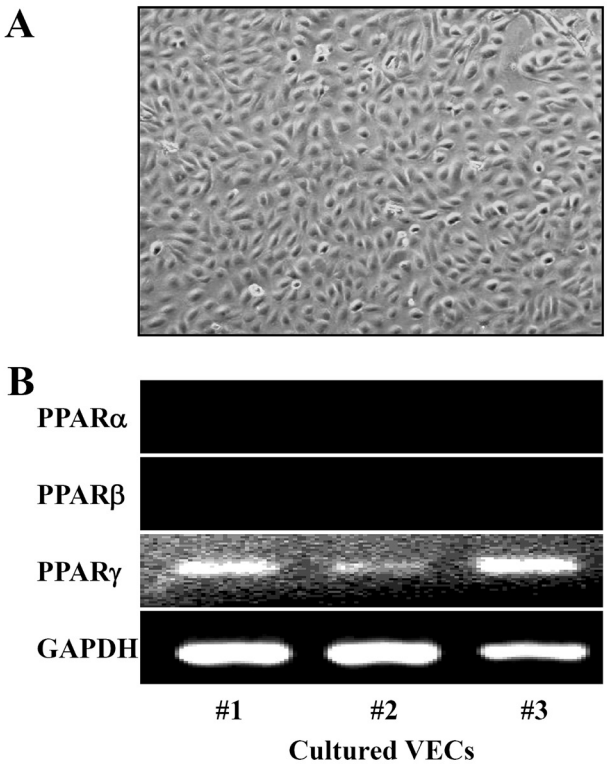

Figure 1 **Expression of peroxisome proliferator-activated receptor (PPAR) γ in rat aortic cultured vascular endothelial cells (VECs)**

A: Under the phase-contrast microscope, a confluent monolayer of cultured VECs (at passage 4) exhibits a "cobblestone" appearance, where cells are in contact with neighboring cells. **B:** reverse transcription polymerase chain reaction (RT-PCR) revealed that PPARγ mRNA, but neither PPARα nor PPARβ mRNA, was constitutively expressed in cultured VECs. Cultured VECs were prepared from the aorta of three different Long Evans Tokushima Otsuka rats (#1, #2, and #3).

Table 1 **Primers for semi-quantitative real-time PCR of mRNA expression in cultured VECs**

Rat cDNA	GenBank accession no.	Primer*		Annealing temperature (℃)	Number of PCR cycles
PPAR α	NM013196	sense	CCTTTTTGTGGCTGCTATAA	57	32
		antisense	TCCCTGCTCTCCTGTATGGG		
PPARb	NM013141	sense	ACGCACCCTTCATCATCCAC	57	32
		antisense	CCAGCAGTCCGTCTTTGTTG		
PPAR γ	AB011365	sense	ACTCCCATTCCTTTGACATC	57	32
		antisense	TCCCCACAGACTCGGCACTC		
GAPDH	M33197	sense	AGTCCATGCCATCACTGCC	58	24
		antisense	ACCACCCTGTTGCTGTAGCC		
PAI-1	M24067	sense	ACCCTCAGCATGTTCATTGC	60	27
		antisense	CTCGTTCACCTCGATCTTGAC		
t-PA	M23697	sense	ATGAAGGGAGAGCTGTTGT	60	30
		antisense	CCCATTGAAGCATCTCGGTT		

The sequences are shown from the 5' to 3'ends.
Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PAI-1, plasminogen activator inhibitor 1; PCR, polymerase chain reaction; PPAR, peroxisome proliferator-activated receptor; t-PA, tissue-type plasminogen activator; VEC, vascular endothelial cell.

Co., Corning, NY). Cell numbers were counted at 0, 24, 48, and 72 h after treatment with 50μM solutions of TMS, TRO, or the control DMSO. To count the viable cells, 5μL of WST-1 was added to each well. After 1-h incubation, absorbance at a wavelength of 415nm (reference wavelength of 550nm) was measured using a MTP-300 microplate reader (Corona Electric Co., Ltd., Hitachinaka, Japan). The blank value (control) was the absorbance of the culture medium without cells.

Statistics

All data are presented as the mean±standard error (SE). Statistical analysis was performed by the Student's unpaired *t*-test or one-way analysis of variance by using Stat View v. 4.5 (Abacus Concepts, CA). $P<0.05$ was defined as statistically significant.

RESULTS

Expression of PPAR mRNA in VECs derived from the aorta of LETO rats

Cultured VECs derived from three different LETO rats were grown to confluence in 10% FBS-Ham's F12-K nutrient medium, and total RNA was extracted to detect mRNA expression of PPARs with RT-PCR. PPARγ mRNA was constitutively expressed in VECs, unlike PPARα and β (Fig. 1B). In contrast, PPARβ mRNA was expressed in adipose tissue from LETO rats (data not shown).

Activation of PPARγ by TMS

To determine the concentration of TMS for the treatment of VECs, PPARγ activity was measured in the presence of various concentrations of TMS. TMS and TRO increased PPARγ activity in a dose-dependent manner and TRO induced higher PPARγ activity than TMS. Data points were fitted with the logistic equation. TMS had an EC_{50} value of 161 μM and TRO had an EC_{50} value of 0.95μM (Fig. 2A). We used the highest concentration of TMS (50μM) that obtained a final solvent (DMSO) concentration of 0.5% in the culture medium.

Effect of TMS on cell proliferation

As shown in Fig. 2B, VECs proliferated with a doubling time of approximately 60 h in 10% FBS-DMEM without TMS and TRO. Furthermore, TRO (10μM) significantly inhibited cell growth ($P<0.05$), but TMS did not. Therefore, the concentration of TMS (50μM) used in this experiment had no effect on VEC proliferation.

Effect of TMS on mRNA expression of PAI-1

The mRNA expression levels of PAI-1 under various TMS concentrations were measured via comparative RT-PCR to evaluate the effect of TMS on fibrinolytic function in VECs (Fig. 3A). Confluent VECs were incubated for 24 h in 0.5% FBS-Ham's

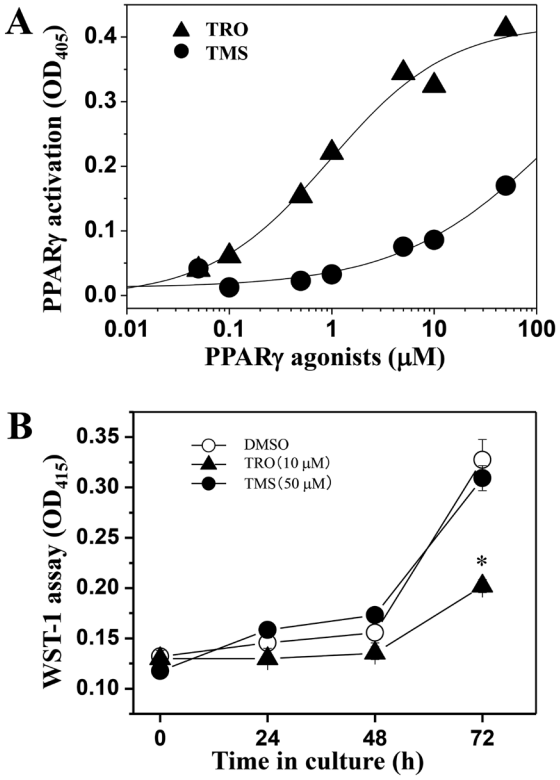

Figure 2 **Effects of telmisartan and troglitazone on PPAR γ activation and cell growth**
A: Concentration-dependent activation of PPARγ by troglitazone (TRO, ▲) and telmisartan (TMS, ●). Serial dilutions for each drug were measured to obtain EC_{50} values by fitting with a logistic equation. **B:** Effects of the PPARγ agonists TRO (▲) and TMS (●) on the cell growth of VECs were compared with those of dimethyl sulfoxide (DMSO, ○). Cell proliferation was determined by a WST-1 assay. Data represent the mean±standard error (SE) for three different experiments. *$P<0.05$ versus DMSO by Student's *t*-test.

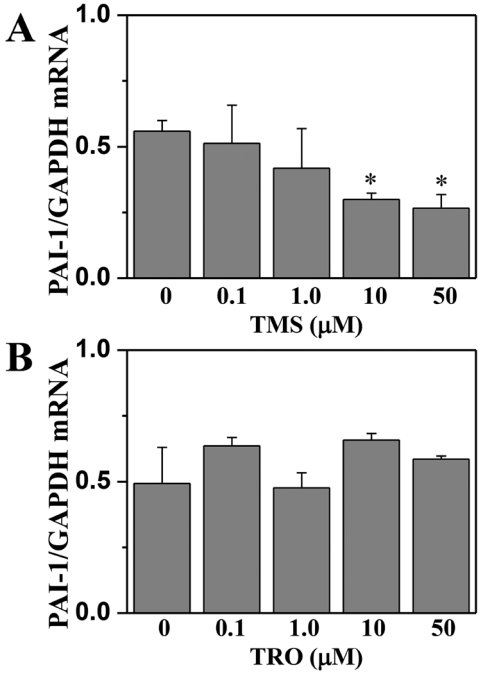

Figure 3 **Effects of TMS and TRO on plasminogen activator inhibitor 1 (PAI-1) mRNA expression in cultured VECs**
Dose-dependent effects on PAI-1/glyceraldehyde-3-phosphate dehydrogenase mRNA expressions of TMS (**A**) and TRO (**B**). Data represent the mean±SE for three different experiments. *$P<0.05$ versus 0μM (DMSO alone) by Student's *t*-test.

F12k medium containing various concentrations of TMS to test the dose-dependent effect of TMS on the mRNA expression of PAI-1. After 24-h treatment, total RNA was extracted and subjected to comparative RT-PCR measurement. TMS decreased PAI-1 mRNA expression in a dose-dependent manner, with an IC50 of 1.2μM and a maximal inhibitory effect of approximately 40%. Furthermore, both 10 and 50μM of TMS significantly decreased PAI-1 mRNA expression (Fig. 3A, $P<0.05$). However, TRO had no effect on PAI-1 mRNA expression (Fig. 3B).

Effect of the PPARγ antagonist GW9662 on the action of TMS

To test whether PPARγ is involved in the downregulation of PAI-1 mRNA by TMS, confluent VECs were incubated for 24 h in 0.5% FBS-Ham's F12k medium containing 50μM of TMS or 10μM of TRO in the presence or absence of GW9662 (0.1μM, Fig. 4A). TMS significantly decreased PAI-1 mRNA expression (Fig. 4B), which is consistent with the data shown in Fig. 3A. Although PAI-1 mRNA expression was decreased by TMS plus GW9662, there was no significant difference from TMS alone. Therefore, GW9662 had no effect on the TMS-induced downregulation of PAI-1 mRNA expression (Fig. 4B). In contrast, GW9662 (control plus GW9662) alone decreased PAI-1 mRNA expression to 45% (Fig. 4B). PPARγ mRNA expression was not altered by TMS in VECs (data not shown). TRO (10μM) had no effect on PAI-1 mRNA expression. The expression

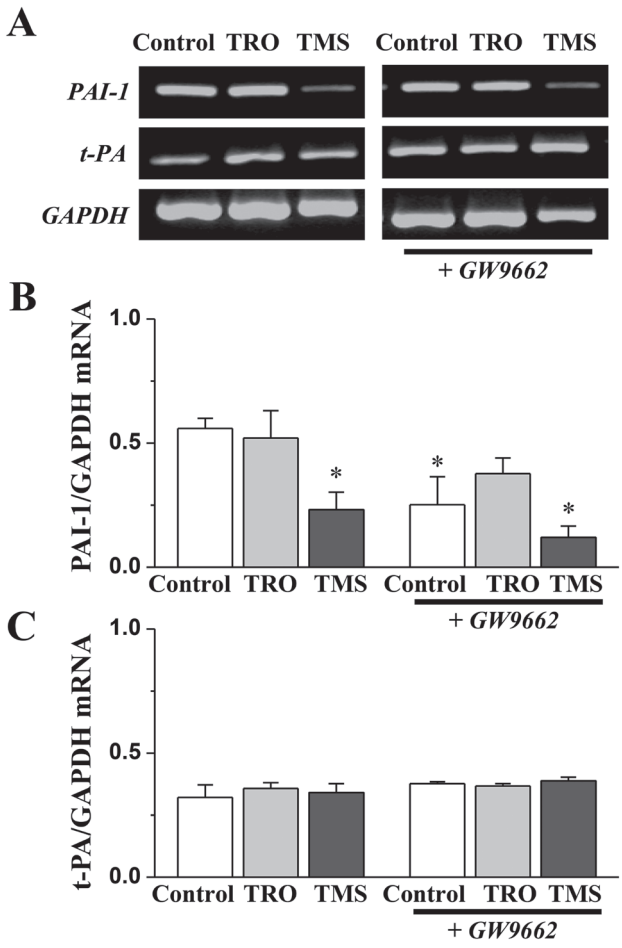

Figure 4 **Involvement of PPARγ in the effects of TMS and TRO on PAI-1 and tissue-type plasminogen activator (t-PA) mRNA expressions in cultured VECs**

A: Agarose electrophoresis showing comparative RT-PCR measurements of PAI-1 and t-PA mRNA expressions after 24-h treatment of cultured VECs with DMSO (control), troglitazone (TRO), and telmisartan (TMS) in the absence (left) or presence (right) of GW9662. **B, C:** Graphic representation of the data summarized in panel A. Data represent the mean±SE from four different experiments. *$P<0.05$ versus control without GW9662 by one-way analysis of variance, followed by multiple comparison tests by Fisher's protected least significant difference.

of t-PA mRNA was not altered by either TMS or TRO, irrespective of their combinations with GW9662 (Fig. 4C).

Effect of the interaction of TMS, TRO, and GW9662 on PPARγ activation

PPARγ activity was measured using the methods described above and depicted in Fig. 2A. TMS increased PPARγ activity in a dose-dependent manner, with an EC_{50} value of 52μM, whereas TRO induced stronger activation of PPARγ, with an EC_{50} value of 3.5μM (Fig. 5A). Interestingly, when 50μM of TMS was mixed with various concentrations of TRO, PPARγ activity decreased at 1-50μM and increased at a TRO concentration of less than 1μM (Fig. 5A). To determine the binding property of TMS to PPARγ, activation of PPARγ by TMS was tested in the presence of the irreversible PPARγ antagonist GW9662. When 50μM of TMS was mixed with various concentrations of GW9662, PPARγ activation by TMS was not inhibited (Fig. 5B, left). When 5μM of TRO was used, GW9662 inhibited PPARγ activity in a dose-dependent manner (Fig. 5B, right).

DISCUSSION

In this study, we showed that the mRNA expression of PAI-1 was significantly decreased by treatment with TMS and that this expression was not abolished by the presence of GW9662. The synthetic PPARγ agonist TRO had no effect on PAI-1 mRNA expression. Furthermore, PAI-1 mRNA expression was significantly decreased by GW9662 (control plus GW9662, Fig. 4B). These data indicate that TMS inhibits PAI-1 mRNA expression

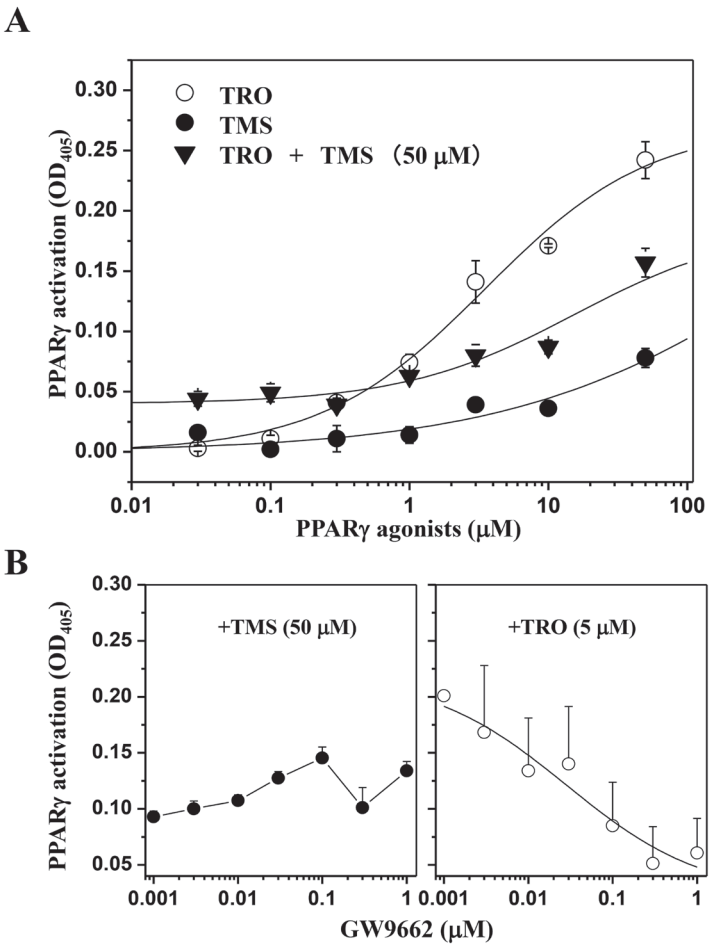

Figure 5 **Comparison of PPAR γ activation between TMS and TRO**
A: Concentration-dependent activation of PPARγ by TRO, TMS and TRO plus TMS. **B:** Effect of the PPARγ antagonist GW9662 on the activation of PPARγ by TMS (left) and TRO (right). Data represent the mean$\pm$SE from three different experiments.

independently of PPARγ activation. Involvement of intrinsic PPARγ agonists, but not extrinsic PPARγ agonists such as TRO, has been suggested in the basal mRNA expression of PAI-1 in VECs[8].

Although it is not yet clear how TMS inhibits PAI-1 mRNA expression, a possible explanation is that TMS acts as an inverse agonist of AT1R. That is, when endogenous activities of AT1R are suppressed by ARBs, AT1R-mediated action is further decreased even without Ang-II. Because Ang-II has been reported to increase PAI-1 mRNA expression in VECs, PAI-1 mRNA expression may be decreased by an inverse agonist activity of TMS for AT1R[12, 13]. In addition, VECs expressed AT1R-associated protein (ATRAP) and AT2R-interacting protein (ATIP), which inhibit AT1R-mediated signals[14, 15]. Our preliminary data showed that olmesartan medoxomil, a potent and selective AT1R antagonist, upregulated ATRAP and ATIP mRNA expressions in VECs (data not shown).

Since TMS has been reported to activate AMP-activated protein kinase (AMPK) and endothelial nitric oxide synthase (eNOS). PPARγ signaling was not involved in TMS-induced these effects[16]. Thus, TMS-induced downregulation of PAI-1 might be mediated by these pathways. Shang *et al* demonstrated that TMS activated AMPK, which phosphorylated and oxidative stress-activated poly (ADP-ribose) polymerase 1 (PARP1) in cultured VECs from diabetic and hypertensive rats. AMPK phosphorylation of PARP1 leads to decreased PARP1 activity, thereby alleviating endothelial dysfunction[17]. Furthermore, It was reported that NO has inhibited the proliferation of human vascular smooth muscle cells through activation of cyclin-dependent kinase inhibitor p21(Waf1/Cip1/Sdi1)[18]. Therefore, TMS could inhibit cell proliferation of VECs through activation of eNOS/p21 pathway (Fig. 2B).

Transcription of PAI-1 may be constitutively upregulated by intrinsic PPARγ agonists such as the long-chain fatty acid 15d-PGJ2[19], but the synthetic PPARγ agonist TRO did not upregulate transcription of PAI-1 in this study. The discrepancy between intrinsic and extrinsic PPARγ agonists is possibly caused by their unique features. For example, different PPARγ agonists lead to differential, but overlapping, patterns of PPARγ target gene expression[20, 21]. Hence, the three-dimensional conformation of the complex of thiazolidinedione with PPARγ was suggested to be different with different ligands, which in turn may recruit somewhat different sets of coactivators or corepressors or do so with altered kinetics[21]. Accordingly, each PPARγ agonist will activate or repress the PAI-1 gene. *In vitro* measurements of PPARγ activity revealed that TMS activated PPARγ via a different mechanism from TRO, and this is supported by our data showing the lack of GW9662 inhibition of PPARγ activation by TMS. Thus, the binding site for TMS on PPARγ must be different from that for TRO. Because TMS inhibited the TRO activation of PPARγ, TMS might allosterically inhibit the activation of PPARγ by TRO[21].

These results suggest that there may be many different binding sites for PPARγ agonists and antagonists on PPARγ[21]. Furthermore, ligands binding to different sites on PPARγ might exert different biological effects mediated by PPARγ[21]. For example, studies with VECs using endogenous PPARγ ligands showed an increase in PAI-1 expression, whereas those using synthetic ligands demonstrated a decreased effect or no effect on PAI-1 expression[22]. This type of activity might modulate the interaction of a PPRE with a target gene, thereby changing its affinity for coactivators and corepressors of PPARγ[4]. PPARγ without ligands is reported to inhibit the transcription of some genes (e.g., glycerol kinase and lectin-like oxidized LDL receptor-1) through an interaction with corepressors that have histone deacetylation activity[23]. When a ligand such as 15d-PGJ_2 binds to PPARγ, nuclear receptor corepressor/silencing mediator of retinoid and thyroid receptors corepressor can be dissociated from PPARγ, allowing the binding of coactivators. Because many coactivators (p300/CBP and SWI/SNF) have histone deacetylation activity, they can upregulate the transcriptions of target genes[5].

In conclusion, TMS inhibited PAI-1 mRNA expression in VECs, and this may play an important role in fibrinolytic function under pathophysiological conditions (e.g., angiogenesis, inflammation, and diabetes mellitus). Further investigation of the modulation of PPARγ by different agonists is necessary to develop a new drug for the regulation of fibrinolytic function in VECs. Further study is needed for confirming the present study results regarding TMS inhibition of PAI-1 mRNA expression using quantitative real time-PCR.

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