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The PPARδ Ligand L-165041 Inhibits VEGF-Induced Angiogenesis, But the Antiangiogenic Effect Is Not Related to PPARδ

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ABSTRACT

Peroxisome proliferator-activated receptor (PPAR)δ is known to be expressed ubiquitously and involved in lipid and glucose metabolism. Recent studies have demonstrated that PPARδ is expressed in endothelial cells (ECs) and plays a potential role in endothelial survival and proliferation. Although PPARα and PPARγ are well recognized to play anti-inflammatory, antiproliferative, and antiangiogenic roles in ECs, the general effect of PPARδ on angiogenesis in ECs remains unclear. Thus, we investigated the effect of the PPARδ ligand L-165041 on vascular EC proliferation and angiogenesis in vitro as well as in vivo. Our data show that L-165041 inhibited VEGF-induced cell proliferation and migration in human umbilical vein ECs (HUVECs). L-165041 also inhibited angiogenesis in the Matrigel plug assay and aortic ring assay. Flow cytometric analysis indicated that L-165041 reduced the number of ECs in the S phase and the expression levels of cell cycle regulatory proteins such as cyclin A, cyclin E, CDK2, and CDK4; phosphorylation of the retinoblastoma protein was suppressed by pretreatment with L-165041. We confirmed whether these antiangiogenic effects of L-165041 were PPARδ-dependent using GW501516 and PPARδ siRNA. GW501516 treatment did not inhibit VEGF-induced angiogenesis, and transfection of PPARδ siRNA did not reverse this antiangiogenic effect of L-165041, suggesting that the antiangiogenic effect of L-165041 on ECs is PPARδ-independent. Together, these data indicate that the PPARδ ligand L-165041 inhibits VEGF-stimulated angiogenesis by suppressing the cell cycle progression independently of PPARδ. This study highlights the therapeutic potential of L-165041 in the treatment of many disorders related to pathological angiogenesis. J. Cell. Biochem. 113: 1947–1954, 2012.

KEY WORDS: ANGIOGENESIS; CELL CYCLE; ENDOTHELIAL CELL; PPARδ

Angiogenesis is the process of new blood vessel formation from preexisting blood vessels, and although physiological angiogenesis is necessary during embryonic development, tissue or organ regeneration, and wound healing, dysregulated angiogenesis is implicated in the pathogenesis of many disorders such as rheumatoid arthritis, diabetes, cardiovascular diseases, and cancers [Folkman, 1995; Ferrara and Kerbel, 2005]. The process of angiogenesis involves complex steps including the activation, migration, and proliferation of endothelial cells (ECs) [Carmeliet, 2003; Karamysheva, 2008], and numerous angiogenic factors have been identified, such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), angiopoietin-1, transforming growth factor (TGF), and vascular endothelium growth factor (VEGF) [Rifkin and Moscatelli, 1989; Nicosia et al., 1994; Suri et al., 1998]. Among these angiogenic factors, VEGF is the most well-known pro-angiogenic factor because of its ability to promote the growth of vascular ECs.

The peroxisome proliferator-activated receptors (PPARs) are ligand-activated nuclear hormone receptors, and their family consists of three structurally similar isoforms: α, δ, and γ. In the classical PPAR pathway, upon binding to ligands, the PPAR forms a heterodimeric complex with the retinoid X receptor. This heterodimeric complex binds to PPAR response elements in the promoter regions of specific target genes that are positively or negatively regulating transcription of genes [Desvergne and Wahli, 1999;

Jin-Hee Park and Kuy-Sook Lee contributed equally to this work.

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Bruemmer et al., 2003; Takano et al., 2004]. Although the roles of PPARα and PPARγ are well-known to be anti-inflammatory, antiproliferative, anti-migrative, and antiangiogenic in ECs, the general effect of PPARδ on angiogenesis in ECs remains unclear [Goetze et al., 2002; Rival et al., 2002; Ward et al., 2004]. PPARδ agonists are not yet in clinical use. Endogenous ligands for PPARδ are fatty acids and prostacyclin [Berger et al., 1999; Xu et al., 1999; Wagner and Wagner, 2010], and synthetic agonists include GW0742, GW2433, L-165041, and GW501516 [Brown et al., 1997; Willson et al., 2000; Brown et al., 2001; Graham et al., 2005]. Previous studies have shown that the PPARδ activator L-165041 inhibits tumor necrosis factor-α-induced EC inflammation (VCAM-1 expression, monocyte adhesion, and MCP-1 secretion). Another report also showed that PPARδ inhibits cell growth of human N/TERT-1 keratinocytes [Burdick et al., 2007]. However, Piqueras et al. demonstrated that GW501516 induced EC proliferation and angiogenesis in vitro and in vivo [Piqueras et al., 2007], and several studies have reported that a natural PPAR ligand such as prostacyclin induce EC angiogenesis [Pola et al., 2004; Stephen et al., 2004]. Thus, examining the effect of PPARδ on ECs is warranted to better understand the role of PPARδ in the cardiovascular system. Here, we investigated the effect of L-165041 on EC proliferation and migration both in vitro and in vivo and elucidated its underlying mechanisms.

According to our data, L-165041 inhibited EC proliferation and migration both in vitro and in vivo. Furthermore, our data indicated that these antiangiogenic effects of L-165041 are closely related to cell cycle arrest, which is accompanied by decreased expression of phospho-Rb, CDK2, CDK4, cyclin A, and cyclin E. In addition, although L-165041 acts as a PPARδ ligand, the antiangiogenic effect of L-165041 was not PPARδ dependent.

MATERIALS AND METHODS

MATERIALS

L-165041 was purchased from Tocris Bioscience (Ellisville, MO), GW501516 was obtained from Alexis Biochemicals (San Diego, CA), and recombinant human VEGF was acquired from R&D Systems (Minneapolis, MN). All chemicals were of analytical grade. Specific antibodies against cyclin A, cyclin E, CDK2, CDK4, and phospho-Rb were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents were purchased from Sigma (St. Louis, MO) unless otherwise noted.

CELL CULTURE AND DRUG TREATMENT

Human umbilical vein ECs (HUVECs) were cultured in EGM-2 (Clonetics, San Diego, CA). Cells at passages between 2 and 10 were used for the study. Subconfluent HUVECs were made quiescent by serum starvation [EBM-2 containing 0.1% fetal bovine serum (FBS)] for 4 h. The cells were pretreated with the PPARδ ligand L-165041 or GW501516 for 6 h followed by VEGF (10 ng/ml) induction.

BrdU INCORPORATION ASSAY

Cells were seeded at 5×10^3 cells/well in 96-well plates and starved for 4 h in EBM-2 containing 0.1% FBS prior to treatment. After the cells were treated with a drug and cultured for additional 24 h, cellular proliferation was determined by a BrdU incorporation assay using a commercial ELISA kit according to the manufacturer's instructions (Roche, Penzberg, Germany). The amount of incorporated BrdU was determined by measuring the absorbance at 450 nm using a microplate reader with SOFTmax PRO software (Molecular Devices, Sunnyvale, CA).

CELL MIGRATION ASSAY: WOUND HEALING ASSAY, AND TRANSWELL ASSAY

HUVEC migration was determined using the wound healing assay. Briefly, HUVECs were seeded into 6-well plates and grown to 90% confluence. The wounds were produced by scraping of the cell layer with sterile cell scraper. For the L-165041 pretreatment group, cells were treated with L-165041 (1–10 μM) 6 h prior to the addition of VEGF (10 ng/ml). The cells were cultured for an additional 24 h before being photographed. HUVEC migration was also assessed using a modified Boyden's chamber method. Cells (5×10^4) were seeded onto the upper surface of an 8-μm pore size chamber (Costar, Inc., Corning, NY). The upper chamber contained media with L-165041 (1–10 μM), and cellular migration was induced by adding VEGF (10 ng/ml) to the lower chamber. After 16 h, non-migrating cells were removed by swabbing with Q-tips and the membrane was fixed in methanol for 30 min. Cells that had migrated were stained with a Diff-Quick staining kit (Kookje Scientific Products, Tokyo, Japan) for 1 h and counted under a light microscope (400×). The number of cells was recorded from at least five fields per well.

TUBE FORMATION ASSAY

Before the HUVECs were plated, the 24-well plates coated with growth factor-reduced basement membrane proteins (Matrigel, 0.3 ml/well; BD Biosciences, Bedford, MA) were solidified at 37°C for 1 h. VEGF and L-165041 were added to the Matrigel solution prior to polymerization. For the angiogenesis assay with HUVECs, the trypsinized cells were plated on the surface of the Matrigel and cultured in serum-deprived medium (8×10^4 cells/well). Tube formation images were analyzed using a service provided by Wimasis [Khoo et al., 2011].

siRNA TRANSFECTION

For transfection, the cells were grown to 20–30% confluence in EGM-2. PPARδ siRNA was delivered into HUVECs using Lipofectamine™ RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. PPARδ siRNA was purchased from Santa Cruz Biotechnology. RT-PCR was conducted 48 h after transfection to evaluate the silencing effect of siRNA on PPARδ expression. A nonspecific control siRNA (Invitrogen) was used as a negative control.

WESTERN BLOT ANALYSIS

For Western blot analysis, VEGF-stimulated cells with or without L-165041 pretreatment were washed with phosphate-buffered saline (PBS) and scraped in 1× protein lysis buffer (Cell Signaling Technology, Danvers, MA). Protein extracts from each group were separated in an SDS–PAGE gel and then transferred onto PVDF membranes. The blots were incubated with appropriate primary antibodies. β-Actin was used to correct for loading errors.

Immunopositive bands were visualized by ECL (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). The relative protein amount was determined by densitometric analysis.

FLOW CYTOMETRY

Cell cycle distribution was determined by flow cytometry. Synchronized HUVECs were pretreated with L-165041 (1 or 5 μM) 6 h prior to the addition of VEGF (10 ng/ml). The cells were harvested 16 h after VEGF addition and washed with PBS. The cells were then incubated with buffer containing 0.1% Triton X-100 and 0.1% trisodium citrate for 30 min. Cells were rinsed with PBS and then stained with 50 μg/ml propidium iodide for 20 min at room temperature. In total, 1×10^4 cells were analyzed with the FACScan system (Becton Dickinson, Franklin Lakes, NJ). At least three independent experiments were performed.

AORTIC RINGS ASSAY AND MATRIGEL PLUG ASSAY

The abdominal aortas were dissected from mice and excess perivascular tissue was removed. Transverse sections (1–2 mm) were made, and the resulting aortic rings were placed on Matrigel-coated wells covered with EGM-2 medium with or without L-165041 (10 μM). Aortic rings were cultured at 37°C and 5% CO_2. On day 5, microvessel outgrowth was photographed. Matrigel (growth factor reduced) in liquid at 4°C was mixed with VEGF (100 ng/ml) with and without L-165041 (10 μM), and the resulting mixture was each injected (0.5 ml) into the abdominal subcutaneous tissue of 6–7-week C57BL/6 mice (Orient Charles River Technology, Seoul, Korea). The mice were killed 7 days after implantation; then the Matrigel plugs were removed, photographed, and examined histologically to determine the extent to which blood vessels had entered. Recovered Matrigel plugs were processed by paraffin embedding followed by H&E staining. All animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals by the Korea National Institute of Health.

STATISTICAL ANALYSIS

Data are presented as the means ± SEM of three or more individual experiments. Significant differences between treatments and controls were identified using a Student's *t*-test or analysis of variance, followed by individual comparisons using Dunnett's test. A *P*-value <0.05 was considered to be statistically significant.

RESULTS

L-165041 INHIBITED VEGF-INDUCED EC PROLIFERATION AND MIGRATION

We first examined the effect of the PPARδ ligand L-165041 on EC proliferation. HUVECs were pretreated with L-165041 for 6 h followed by VEGF (10 ng/ml) induction. DNA synthesis was increased by VEGF treatment but this VEGF-induced cell proliferation was abrogated by L-165041 (1–10 μM) in a dose-dependent manner (Fig. 1A). To investigate the outcome of the PPARδ ligand on HUVEC migration, we examined the effect of L-165041 using a wound healing assay. VEGF-induced cellular migration was inhibited after 6 h of L-165041 (1 or 5 μM) pretreatment (Fig. 1B). This anti-migrative effect of L-165041 was also confirmed by Transwell assays. Cells that had migrated were increased by VEGF treatment, and these increased cells were significantly decreased by L-165041 (1 or 5 μM) treatment (Fig. 1C). Next, we examined whether L-165041 affected EC tube formation using a Matrigel assay. As shown in Figure 1D, HUVECs cultured on Matrigel containing VEGF (10 ng/ml) were stretched and elongated, forming a capillary-like structure within 4 h, but VEGF-induced tube formation was attenuated by L-165041 (1 or 5 μM).

EFFECT OF L-165041 ON EX VIVO AND IN VIVO ANGIOGENESIS

Based on the in vitro effects of L-165041 on angiogenesis, we performed an aortic rings assay in mice to test whether L-165041 affects EC growth ex vivo. Mice aortic rings were placed on a Matrigel plug and incubated with or without L-165041 (10 μM). Aortic rings treated with L-165041 showed significantly attenuated EC outgrowth compared to control aortic rings (Fig. 2A). To determine the effect of L-165041 on VEGF-induced angiogenesis in vivo, a Matrigel plug assay was conducted via histological examination. The mice were killed 7 days after implantation and the Matrigel plugs were processed by paraffin embedding followed by H&E staining. Blood vessel formation was induced in Matrigel containing VEGF (10 ng/ml). This effect was decreased in Matrigel to which L-165041 was added (Fig. 2B).

EFFECT OF L-165041 ON CELL CYCLE PROGRESSION AND ITS REGULATORY PROTEINS IN HUVECs

To further examine the underlying mechanisms of the antiproliferative and anti-migrative effect of L-165041, we performed a cell cycle analysis using FACS. According to our data, 16 h of VEGF treatment induced significant S-phase transition compared to the control group. The L-165041 pretreatment significantly suppressed this VEGF-induced S-phase transition (Fig. 3A and Supplemental Fig. S1A). To determine the molecular basis for the cell cycle arrest induced by L-165041, we examined the expression of multiple cell cycle regulatory proteins by Western blotting. Compared to control cells, cells treated with VEGF showed significantly increased phosphorylation of Rb, CDK2, CDK4, cyclin A, and cyclin E, which plays an important role in cell cycle progression. These regulatory proteins were significantly suppressed by pretreatment with 5 μM L-165041 (Fig. 3B and Supplemental Fig. S1B). These findings suggest that L-165041 negatively affects cell cycle progression in VEGF-activated HUVECs.

L-165041 INHIBITS VEGF-INDUCED ANGIOGENESIS PPARδ INDEPENDENTLY

To determine whether the observed effect of L-165041 was PPARδ-dependent, we performed further experiments using another PPARδ ligand GW501516 and PPARδ siRNA. Pretreatment of GW501516 (100 nM) did not inhibit VEGF-induced proliferation and migration in HUVECs (Fig. 4A and upper panel of B). Next, HUVECs were transfected with PPARδ siRNA (20 nM) for 48 h, and we evaluated the silencing effect of siRNA on PPARδ expression using RT-PCR and Western blotting (Supplemental Fig. S2). L-165041 inhibited VEGF-induced EC migration in cells transfected with control siRNA (data not shown). And as shown in lower panel of Figure 4B and C, PPARδ siRNA transfection failed to reverse the antiangiogenic effect

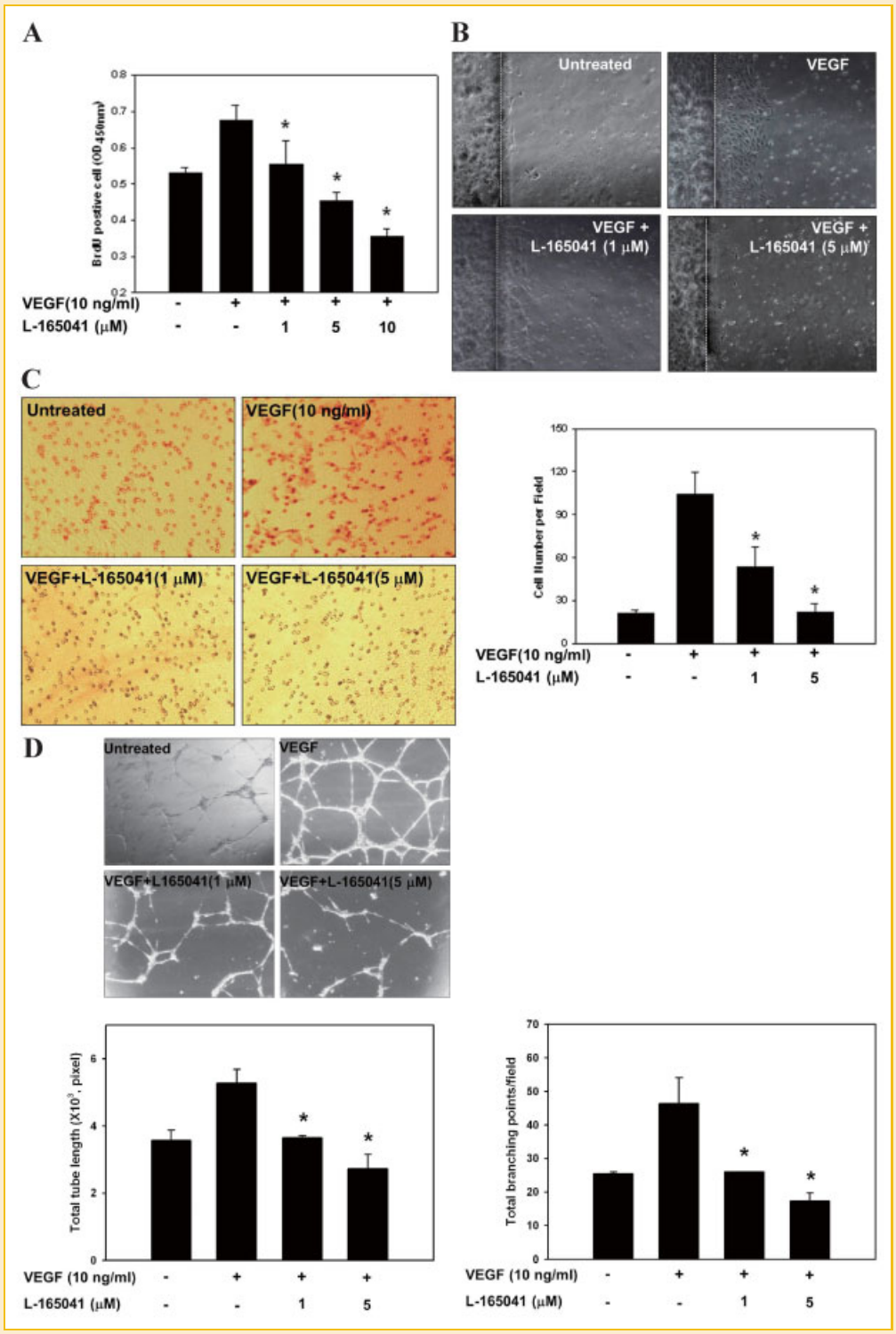

Fig. 1. L-165041 inhibited VEGF-induced EC proliferation and migration. A: HUVECs were pretreated with various concentrations of L-165041 (1–10 μM) 6 h prior to the 24 h of VEGF (10 ng/ml) treatment. DNA synthesis was measured using the BrdU incorporation assay. B: Representative photomicrographs of wound healing assays. A wound was made using near confluent HUVECs in a 6-well culture dish and a scraper. HUVECs were pretreated with L-165041 (1 or 5 μM) for 6 h before the induction of cellular migration with VEGF (10 ng/ml). A photograph was taken after 24 h of incubation. C: HUVECs were cultured in a Boyden chamber with VEGF (10 ng/ml) in the lower chamber and either 1 or 5 μM of L-165041 in the upper chamber. After 16 h, cells that had migrated were stained with a Diff-Quick staining kit, and then a photograph was taken. Cellular migration was determined by counting cells that migrated through the pores. Migrated-cell numbers are represented in a bar graph format. D: Upper panel, Representative photomicrographs of tube formation assays. HUVEC cells were pretreated with or without L-165041 (1 or 5 μM) for 6 h before the cells were seeded on Matrigel. The trypsinized cells were plated on the surface of growth factor-reduced Matrigel (8×10^4 cells/well) and treated with VEGF (10 ng/ml) or L-165041 (1 or 5 μM). A photograph was taken after 4 h of incubation; Lower panel, Analyzed tube formation are represented in a bar graph format. Data are represented as the average of at least three independent experiments ± SEM. The data were statistically evaluated by analysis of variance, followed by individual comparisons using Dunnett's test. $^*P < 0.05$. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

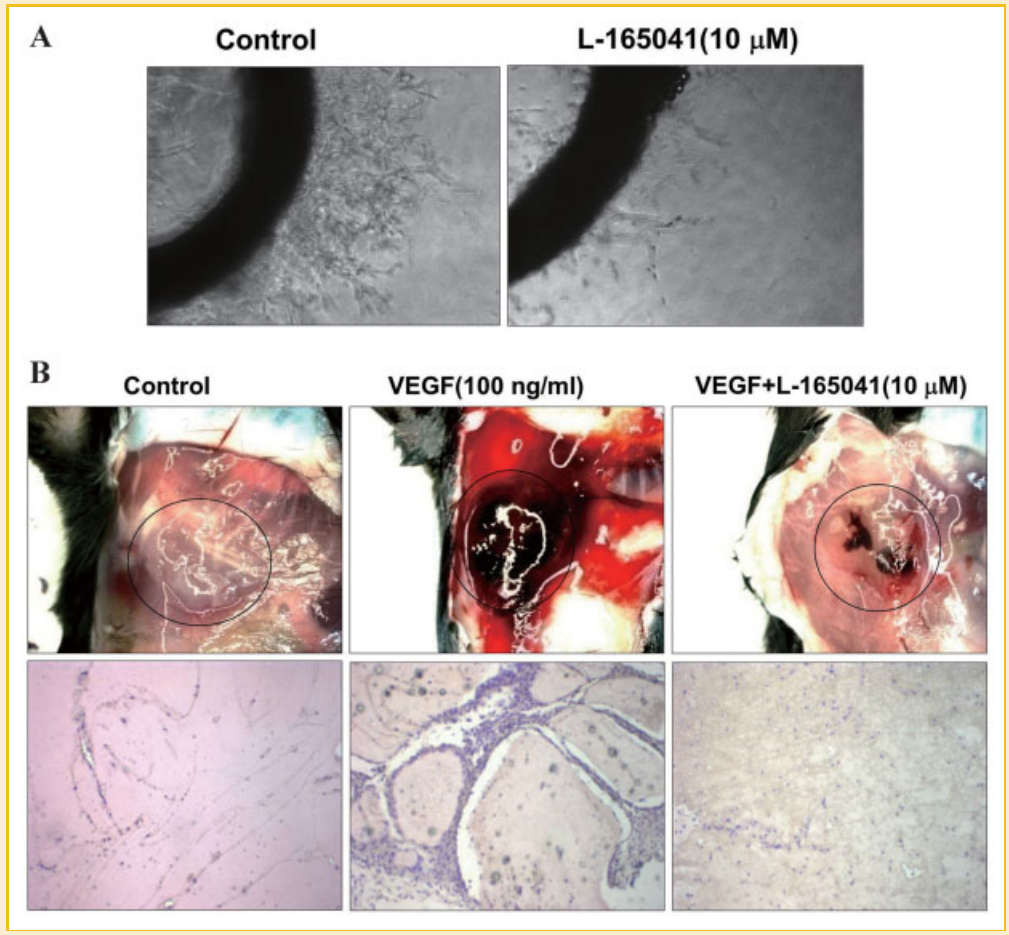

Fig. 2. L-165041 inhibited VEGF-induced angiogenesis ex vivo and in vivo. A: The abdominal aortas were dissected from mice and excess perivascular tissue was removed. The aortic rings were placed on Matrigel-coated wells and incubated in the absence or presence of L-165041 (10 μM). After 5 days of incubation, microvessel outgrowth was photographed. Pictures are representative photographs for endothelial cell sprouts formed from the aortic ring segments. B: C57BL/6 mice (three per group) were injected subcutaneously with or without L-165041 (10 μM), together with Matrigel (growth factor reduced) containing VEGF (100 ng/ml), and 40 U heparin/ml at 4°C. The mice were euthanized 7 days after implantation. Upper panel, photograph of the gross appearance of the plugs; lower panel, H&E staining for histological analysis. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

of L-165041, which inhibited EC migration as well as the expression of the cell cycle-related protein.

DISCUSSION

Recent studies have demonstrated that PPARδ is widely expressed in several different cell types, including ECs, and plays a potential role in anti-inflammatory and antiproliferative processes [Kliewer et al., 1994; Wang, 2008]. Numerous reports have indicated that PPARδ inhibits cell growth in different cancer cell lines, keratinocytes, and ECs [Kim et al., 2005, 2006; Liou et al., 2006; Burdick et al., 2007]. Conversely, other studies have reported that PPARδ induced EC and endothelial progenitor cell proliferation and angiogenesis [Piqueras et al., 2007; Han et al., 2008], as well as accelerated cancer cell growth [Gupta et al., 2004; Stephen et al., 2004]. Although the effect of PPARδ on cellular proliferation remains unclear, few studies have investigated the role and mechanism of PPARδ in cardiovascular disease. Therefore, we examined the association between PPARδ and vascular EC proliferation and its underlying mechanisms using L-165041.

Our data showed that VEGF-induced cell proliferation was significantly inhibited by L-165041 in HUVECs and that L-165041 also significantly inhibited HUVEC migration. In addition, L-165041 blocked endothelial differentiation into tubelike structures in vitro and VEGF-induced angiogenesis in vivo. These results demonstrate that the antiangiogenic action is coupled with distinct alterations of cell cycle progression.

During cell cycle progression, the progression from G1 to S phase is closely linked to the activation of cell cycle regulatory proteins such as cyclin-dependent kinases (CDKs) [Lavoie et al., 1996]. In particular, the cyclin D1/CDK4 and cyclin E/CDK2 complexes are essential for entering S phase [Wei et al., 1997]. Induction of cyclin A expression and cyclin/CDK complexes mediate retinoblastoma protein (Rb) phosphorylation in late G1 phase, and hyperphosphorylated Rb induces the release of the transcription factor E2F, resulting in cell cycle progression to S phase [Zhan et al., 2002; Andres, 2004; Fasciano et al., 2005; Nie et al., 2006]. Our data also

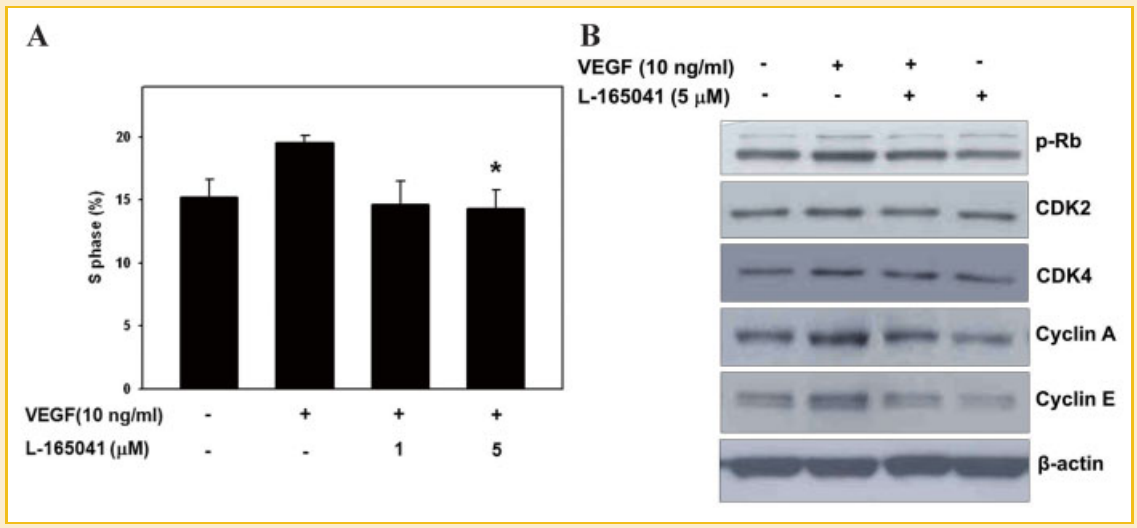

Fig. 3. L-165041 negatively affected cell cycle progression in VEGF-activated HUVECs. A: Cell cycle distribution was determined by FACS analysis. HUVECs were pretreated with or without L-165041 (1 or 5 μM) 6 h prior to the addition of VEGF (10 ng/ml). The cells were harvested 16 h after VEGF addition. In total, 1×10^4 cells were analyzed with FACScan (Becton Dickinson). Comparison of the percentage of cells in S phase. Data are represented as the average of at least three independent experiments ± SEM. $^*P < 0.05$. B: HUVECs were pretreated with L-165041 (5 μM) for 6 h before the induction of cellular stimulation with VEGF (10 ng/ml). After 24 h, cell lysates were prepared and the expression levels of p-Rb, cyclins, and CDKs were determined by Western blotting. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

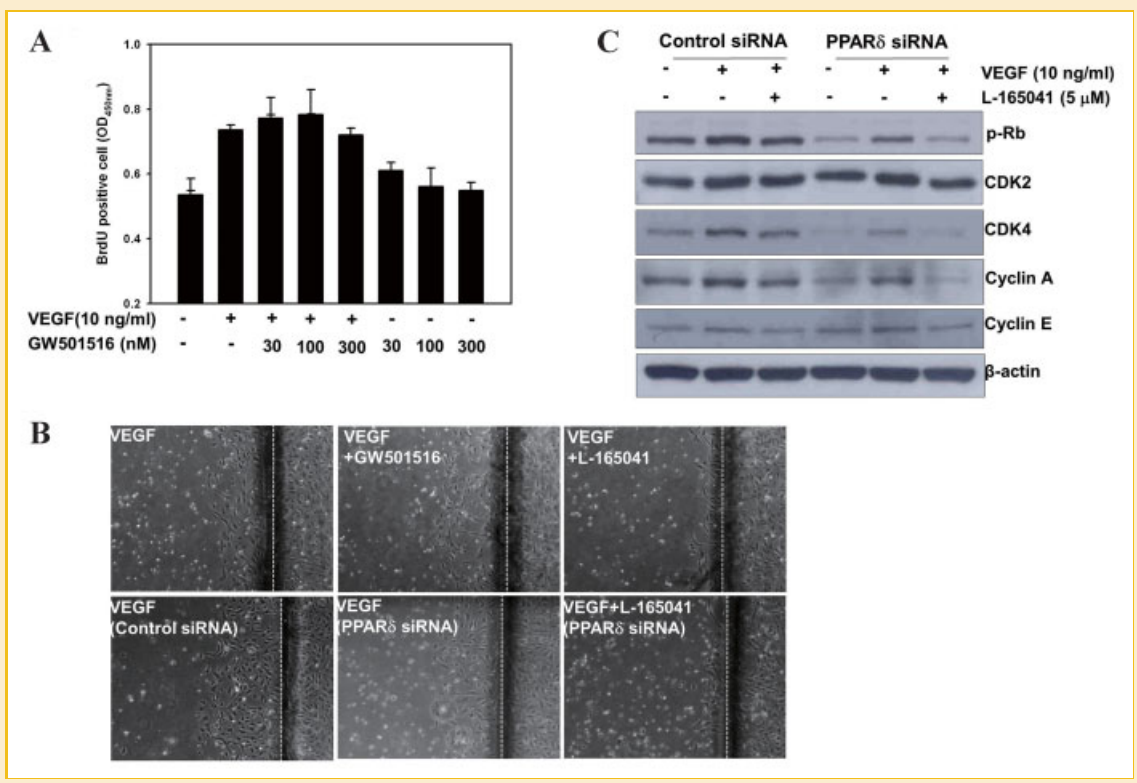

Fig. 4. L-165041 inhibited PPARδ-independent, VEGF-induced angiogenesis. A: HUVECs were pretreated with various concentrations of GW501516 (30–300 nM) 6 h prior to the 24 h of VEGF (10 ng/ml) treatment. DNA synthesis was measured with a BrdU incorporation assay. B: Representative photomicrographs of wound healing assays. Upper panel, HUVECs were pretreated with GW501516 (100 nM) or L-165041 (5 μM) for 6 h before the induction of cellular migration with VEGF (10 ng/ml); lower panel, 48 h after transfection with PPARδ siRNA (20 nM) in HUVECs, cells were pretreated with L-165041 (5 μM) for 6 h before the induction of cellular migration with VEGF (10 ng/ml). A photograph was taken after 24 h of incubation. C: Forty-eight hours after transfection, cells were pretreated with L-165041 (5 μM) for 6 h before the induction with VEGF (10 ng/ml). After 24 h, cell lysates were prepared and the expression levels of p-Rb, cyclins, and CDKs were determined by Western blotting. Data are represented as the average of at least three independent experiments ± SEM. The data were statistically evaluated by analysis of variance, followed by individual comparisons using Dunnett's test. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

show that L-165041 decreased the expression of cyclin A, CDK4, cyclin E, and CDK2, as well as the subsequent phosphorylation of Rb. These results suggest that L-165041 causes cell cycle arrest by downregulating cell cycle regulators. These findings are consistent with our previous report stating that L-165041 inhibited cell growth in vascular smooth muscle cells [Lim et al., 2009]. Furthermore, L-165041 attenuated VEGF-induced VEGFR phosphorylation, and the downstream components of VEGF signaling, ERK1/2 MAP kinase pathway but not PI3K-Akt signaling pathway (Supplemental Fig. S3). Because the previous studies reported that PPARδ ligand increased VEGF mRNA expression or secretion [Fauconnet et al., 2002; Piqueras et al., 2007], we performed the ELISA assay for the determination of the effect of L-165041 on VEGF secretion. According to our data, L-165041 did not affect VEGF secretion (Supplemental Fig. S4). These results indicate that L-165041 pretreatment specifically inhibits the VEGFR-ERK1/2 pathway, thereby induces subsequent cell cycle arrest, without modulating VEGF secretion, under our experimental conditions. In an unpublished study, we observed that treatment of L-165041 increased apoptotic cells compared to VEGF-treated ECs, which was observed in a population of sub-G1 cells and FITC–annexin-V positive cells. Therefore, we hypothesize that the antiangiogenic effects of L-165041 were caused by inhibition of cell cycle progression and induction of apoptosis in HUVECs.

In further experiments, we confirmed whether these antiangiogenic effects of L-165041 were PPARδ-dependent using GW501516 and PPARδ siRNA. GW501516, which is a more selective PPARδ ligand compared to L-165041 ($EC_{50} = 1.1$ nM), is currently in a phase II clinical trial for dyslipidemia [Sznaidman et al., 2003]. Notably, pretreatment with another PPARδ ligand, GW501516, did not inhibit VEGF-induced EC proliferation, migration, and expression of the cell cycle-related protein (data not shown). In addition, transfection of PPARδ siRNA in HUVECs did not reverse this antiangiogenic effect of L-165041, indicating that the antiangiogenic effect of L-165041 on ECs was PPARδ-independent. Thus, we can speculate that the antiangiogenic effects of L-165041 seem to be only characteristic of L-165041, and not PPARδ. Similar results have been reported for PPARγ; that is, many thiazolidinedione (TZD, synthetic ligand of PPARγ) derivatives may produce pharmacological effects independently of PPARγ. These PPARγ-independent signals were demonstrated in numerous reports [Lee et al., 2006; Al Alem et al., 2011; Hu et al., 2011]. At present, the PPARδ-independent pathways activated by L-165041 have not been fully defined and future studies are needed.

In conclusion, we showed that the PPARδ ligand L-165041 inhibits EC proliferation and migration by suppressing the cell cycle progression of a PPARδ-independent pathway. The present data highlight the need for further studies and suggest a therapeutic potential of L-165041 in the treatment of many disorders related to pathological angiogenesis.

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REFERENCES

Al Alem L, Southard RC, Kilgore MW, Curry TE. 2011. Specific thiazolidinediones inhibit ovarian cancer cell line proliferation and cause cell cycle arrest in a PPARgamma independent manner. PLoS ONE 6:e16179. DOI: 10.1371/journal.pone.0016179

Andres V. 2004. Control of vascular cell proliferation and migration by cyclin-dependent kinase signalling: New perspectives and therapeutic potential. Cardiovasc Res 63:11–21.

Berger J, Leibowitz MD, Doebber TW, Elbrecht A, Zhang B, Zhou G, Biswas C, Cullinan CA, Hayes NS, Li Y, Tanen M, Ventre J, Wu MS, Berger GD, Mosley R, Marquis R, Santini C, Sahoo SP, Tolman RL, Smith RG, Moller DE. 1999. Novel peroxisome proliferator-activated receptor (PPAR) gamma and PPAR-delta ligands produce distinct biological effects. J Biol Chem 274:6718–6725.

Brown PJ, Smith-Oliver TA, Charifson PS, Tomkinson NC, Fivush AM, Sternbach DD, Wade LE, Orband-Miller L, Parks DJ, Blanchard SG, Kliewer SA, Lehmann JM, Willson TM. 1997. Identification of peroxisome proliferator-activated receptor ligands from a biased chemical library. Chem Biol 4:909–918.

Brown PJ, Stuart LW, Hurley KP, Lewis MC, Winegar DA, Wilson JG, Wilkison WO, Ittoop OR, Willson TM. 2001. Identification of a subtype selective human PPARalpha agonist through parallel-array synthesis. Bioorg Med Chem Lett 11:1225–1227.

Bruemmer D, Yin F, Liu J, Berger JP, Sakai T, Blaschke F, Fleck E, Van Herle AJ, Forman BM, Law RE. 2003. Regulation of the growth arrest and DNA damage-inducible gene 45 (GADD45) by peroxisome proliferator-activated receptor gamma in vascular smooth muscle cells. Circ Res 93:e38–e47.

Burdick AD, Bility MT, Girroir EE, Billin AN, Willson TM, Gonzalez FJ, Peters JM. 2007. Ligand activation of peroxisome proliferator-activated receptor-beta/delta (PPAR beta/delta) inhibits cell growth of human N/TERT-1 keratinocytes. Cell Signal 19:1163–1171.

Carmeliet P. 2003. Angiogenesis in health and disease. Nat Med 9:653–660.

Desvergne B, Wahli W. 1999. Peroxisome proliferator-activated receptors: Nuclear control of metabolism. Endocr Rev 20:649–688.

Fasciano S, Patel RC, Handy I, Patel CV. 2005. Regulation of vascular smooth muscle proliferation by heparin: Inhibition of cyclin-dependent kinase 2 activity by p27(kip1). J Biol Chem 280:15682–15689.

Fauconnet S, Lascombe I, Chabannes E, Adessi GL, Desvergne B, Wahli W, Bittard H. 2002. Differential regulatin of vascular endothelial growth factor expression by peroxisome proliferator-activated receptors in bladder cancer cells. J Biol Chem 277:23534–23543.

Ferrara N, Kerbel RS. 2005. Angiogenesis as a therapeutic target. Nature 438:967–974.

Folkman J. 1995. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat Med 1:27–31.

Goetze S, Eilers F, Bungenstock A, Kintscher U, Stawowy P, Blaschke F, Graf K, Law RE, Fleck E, Grafe M. 2002. PPAR activators inhibit endothelial cell migration by targeting Akt. Biochem Biophys Res Commun 293:1431–1437.

Graham TL, Mookherjee C, Sucking KE, Palmer CN, Patel L. 2005. The PPARdelta agonist GW0742X reduces atherosclerosis in LDLR(-/-) mice. Atherosclerosis 181:29–37.

Gupta RA, Wang D, Katkuri S, Wang H, Dey SK, DuBois RN. 2004. Activation of nuclear hormone receptor peroxisome proliferator-activated receptor-delta accelerates intestinal adenoma growth. Nat Med 10:245–247.

Han JK, Lee HS, Yang HM, Hur J, Jun SI, Kim JY, Cho CH, Koh GY, Peters JM, Park KW, Cho HJ, Lee HY, Kang HJ, Oh BH, Park YB, Kim HS. 2008. Peroxisome proliferator-activated receptor-delta agonist enhances vasculogenesis by regulating endothelial progenitor cells through genomic and nongenomic activations of the phosphatidylinositol 3-kinase/Akt pathway. Circulation 118:1021–1033.

Hu Y, Liu HB, Simpson RW, Dear AE. 2011. PPARgamma-independent thiazolidinedione-mediated inhibition of NUR77 expression in vascular endothelial cells. J Endocrinol 208:R1–R7.

Karamysheva AF. 2008. Mechanisms of angiogenesis. Biochemistry (Mosc) 73:751–762.

Khoo CP, Micklem K, Watt SM. 2011. A comparison of methods for quantifying angiogenesis in the Matrigel assay in vitro. Tissue Eng Part C Methods 17:895–906.

Kim DJ, Murray IA, Burns AM, Gonzalez FJ, Perdew GH, Peters JM. 2005. Peroxisome proliferator-activated receptor-beta/delta inhibits epidermal cell proliferation by down-regulation of kinase activity. J Biol Chem 280:9519–9527.

Kim DJ, Bility MT, Billin AN, Willson TM, Gonzalez FJ, Peters JM. 2006. PPARbeta/delta selectively induces differentiation and inhibits cell proliferation. Cell Death Differ 13:53–60.

Kliewer SA, Forman BM, Blumberg B, Ong ES, Borgmeyer U, Mangelsdorf DJ, Umesono K, Evans RM. 1994. Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. Proc Natl Acad Sci USA 91:7355–7359.

Lavoie JN, L'Allemain G, Brunet A, Muller R, Pouyssegur J. 1996. Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. J Biol Chem 271:20608–20616.

Lee KS, Park JH, Lee S, Lim HJ, Jang Y, Park HY. 2006. Troglitazone inhibits endothelial cell proliferation through suppression of casein kinase 2 activity. Biochem Biophys Res Commun 346:83–88.

Lim HJ, Lee S, Park JH, Lee KS, Choi HE, Chung KS, Lee HH, Park HY. 2009. PPAR delta agonist L-165041 inhibits rat vascular smooth muscle cell proliferation and migration via inhibition of cell cycle. Atherosclerosis 202:446–454.

Liou JY, Lee S, Ghelani D, Matijevic-Aleksic N, Wu KK. 2006. Protection of endothelial survival by peroxisome proliferator-activated receptor-delta mediated 14-3-3 upregulation. Arterioscler Thromb Vasc Biol 26:1481–1487.

Nicosia RF, Nicosia SV, Smith M. 1994. Vascular endothelial growth factor, platelet-derived growth factor, and insulin-like growth factor-1 promote rat aortic angiogenesis in vitro. Am J Pathol 145:1023–1029.

Nie L, Wise M, Peterson D, Meydani M. 2006. Mechanism by which avenanthramide-c, a polyphenol of oats, blocks cell cycle progression in vascular smooth muscle cells. Free Radic Biol Med 41:702–708.

Piqueras L, Reynolds AR, Hodivala-Dilke KM, Alfranca A, Redondo JM, Hatae T, Tanabe T, Warner TD, Bishop-Bailey D. 2007. Activation of PPARbeta/delta induces endothelial cell proliferation and angiogenesis. Arterioscler Thromb Vasc Biol 27:63–69.

Pola R, Gaetani E, Flex A, Aprahamian TR, Bosch-Marce M, Losordo DW, Smith RC, Pola P. 2004. Comparative analysis of the in vivo angiogenic properties of stable prostacyclin analogs: A possible role for peroxisome proliferator-activated receptors. J Mol Cell Cardiol 36:363–370.

Rifkin DB, Moscatelli D. 1989. Recent developments in the cell biology of basic fibroblast growth factor. J Cell Biol 109:1–6.

Rival Y, Beneteau N, Taillandier T, Pezet M, Dupont-Passelaigue E, Patoiseau JF, Junquero D, Colpaert FC, Delhon A. 2002. PPARalpha and PPARdelta activators inhibit cytokine-induced nuclear translocation of NF-kappaB and expression of VCAM-1 in EAhy926 endothelial cells. Eur J Pharmacol 435:143–151.

Stephen RL, Gustafsson MC, Jarvis M, Tatoud R, Marshall BR, Knight D, Ehrenborg E, Harris AL, Wolf CR, Palmer CN. 2004. Activation of peroxisome proliferator-activated receptor delta stimulates the proliferation of human breast and prostate cancer cell lines. Cancer Res 64:3162–3170.

Suri C, McClain J, Thurston G, McDonald DM, Zhou H, Oldmixon EH, Sato TN, Yancopoulos GD. 1998. Increased vascularization in mice overexpressing angiopoietin-1. Science 282:468–471.

Sznaidman ML, Haffner CD, Maloney PR, Fivush A, Chao E, Goreham D, Sierra ML, LeGrumelec C, Xu HE, Montana VG, Lambert MH, Willson TM, Oliver WR, Sternbach DD. 2003. Novel selective small molecule agonists for peroxisome proliferator-activated receptor delta (PPARdelta)—synthesis and biological activity. Bioorg Med Chem Lett 13:1517–1521.

Takano H, Hasegawa H, Zou Y, Komuro I. 2004. Pleiotropic actions of PPAR gamma activators thiazolidinediones in cardiovascular diseases. Curr Pharm Des 10:2779–2786.

Wagner KD, Wagner N. 2010. Peroxisome proliferator-activated receptor beta/delta (PPARbeta/delta) acts as regulator of metabolism linked to multiple cellular functions. Pharmacol Ther 125:423–435.

Wang N. 2008. PPAR-delta in vascular pathophysiology. PPAR Res 2008:164163, 10. DOI: 10.1155/2008/164163

Ward JE, Gould H, Harris T, Bonacci JV, Stewart AG. 2004. PPAR gamma ligands, 15-deoxy-delta12,14-prostaglandin J2 and rosiglitazone regulate human cultured airway smooth muscle proliferation through different mechanisms. Br J Pharmacol 141:517–525.

Wei GL, Krasinski K, Kearney M, Isner JM, Walsh K, Andres V. 1997. Temporally and spatially coordinated expression of cell cycle regulatory factors after angioplasty. Circ Res 80:418–426.

Willson TM, Brown PJ, Sternbach DD, Henke BR. 2000. The PPARs: From orphan receptors to drug discovery. J Med Chem 43:527–550.

Xu HE, Lambert MH, Montana VG, Parks DJ, Blanchard SG, Brown PJ, Sternbach DD, Lehmann JM, Wisely GB, Willson TM, Kliewer SA, Milburn MV. 1999. Molecular recognition of fatty acids by peroxisome proliferator-activated receptors. Mol Cell 3:397–403.

Zhan Y, Kim S, Yasumoto H, Namba M, Miyazaki H, Iwao H. 2002. Effects of dominant-negative c-Jun on platelet-derived growth factor-induced vascular smooth muscle cell proliferation. Arterioscler Thromb Vasc Biol 22:82–88.