Molecular Cardiology

Peroxisome Proliferator–Activated Receptor-δ Agonist Enhances Vasculogenesis by Regulating Endothelial Progenitor Cells Through Genomic and Nongenomic Activations of the Phosphatidylinositol 3-Kinase/Akt Pathway

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Background—Despite the therapeutic potential of endothelial progenitor cells (EPCs) in ischemic vascular diseases, their insufficient numbers limit clinical applications. Peroxisome proliferator–activated receptor (PPAR)-δ belongs to the nuclear hormone receptor superfamily, and its functions in various tissues and cells are almost unexplored, especially with respect to vascular biology.

Methods and Results—PPAR-δ activation in EPCs phosphorylated Akt, and this phosphorylation was mediated not only by genomic but also by nongenomic pathways through interaction with the regulatory subunit of phosphatidylinositol 3-kinase. PPAR-δ activation with agonist (GW501516 or L-165041) increased the proliferation of human EPCs and protected them from hypoxia-induced apoptosis. In addition, PPAR-δ activation enhanced EPC functions, such as transendothelial migration, and tube formation. These actions by PPAR-δ activation in EPCs were dependent on the phosphatidylinositol 3-kinase/Akt pathway. In ischemic hindlimb of mice models, transplantation of PPAR-δ agonist–treated human or mouse EPCs enhanced blood flow recovery to ischemic limbs compared with vehicle-treated EPCs. In EPCs from PPAR-δ–knockout mice, however, treatment with PPAR-δ agonist did not enhance in vivo vasculogenic potential. Systemic administration of PPAR-δ agonist increased hematopoietic stem cells in bone marrow and EPCs in peripheral blood, leading to improved vasculogenesis with incorporation of bone marrow–derived cells to new vessels in a corneal neovascularization model and limb salvage with better blood flow in an ischemic hindlimb model.

Conclusions—The results of our study suggest that PPAR-δ agonist has therapeutic vasculogenic potential for the treatment of ischemic cardiovascular diseases. (*Circulation*. 2008;118:1021-1033.)

Key Words: progenitor cells, endothelial ■ peroxisome proliferator–activated receptors ■ vasculogenesis

New vessel formation is an essential compensatory response to provide oxygen to ischemic tissues. Accumulating evidence now indicates that bone marrow (BM)–derived endothelial progenitor cells (EPCs) play a crucial role in adult blood vessel formation.[1] Despite the potential therapeutic applications of EPCs in tissue ischemia, its use in clinical practice is limited because of its scarcity in the circulating blood.[2]

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The peroxisome proliferator–activated receptors (PPARs) form a subfamily of the nuclear hormone receptor superfam-

ily.[3] Three isoforms have been identified thus far, ie, PPAR-α, PPAR-γ, and PPAR-δ. Recent data have implicated PPAR-γ ligands in angiogenesis modulation[4] and EPC biology.[5] Most studies conducted on the topic have concluded that PPAR-γ activation inhibits angiogenesis.[4] Therefore, PPAR-γ modulation may be a therapeutic target in numerous pathologies involving excessive angiogenesis, such as cancer.[6]

By contrast, because of its ubiquitous expression and the delayed development of selective ligands, PPAR-δ is the least understood PPAR subtype.[7] Most studies have associated PPAR-δ activation with its metabolic effects. Interestingly, some evidence indicates that the activation of PPAR-δ stimulates angiogenesis. PPAR-δ activation was shown to induce the proliferation of endothelial cells (ECs)[8,9] and protect them from apoptosis.[10] However, few studies have systematically evaluated the implications of PPAR-δ activation in vascular biology. In particular, the role of PPAR-δ in vasculogenesis and its effect on EPCs are virtually unknown.

Thus, in the present study, we studied the effects of PPAR-δ activation on the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway in detail and showed that both genomic and nongenomic activations occurred. Next, we evaluated the proliferation, apoptosis, and various functions of EPCs after PPAR-δ activation. Finally, the in vivo effect of PPAR-δ activation was also investigated in 2 ways, ie, local delivery of agonist-treated EPCs and systemic administration of agonist.

Methods

Expanded methods are available in the online-only Data Supplement.

Cell Culture

All human aspects of this study were approved by the institutional review board of Seoul National University Hospital. For early EPC cultures, 50 mL of peripheral blood was obtained from healthy volunteers with informed consent. The mononuclear cells (MNCs) were fractionated with Ficoll as described previously,[11,12] and isolated MNCs were resuspended with the use of an EGM-2MV BulletKit system (Clonetics) consisting of endothelial basal medium, 5% fetal bovine serum (FBS), human epidermal growth factor, vascular endothelial growth factor, human fibroblast growth factor-B, insulin-like growth factor-1, ascorbic acid, and heparin; 1×10^7 MNCs per well were seeded on 2% fibronectin-coated (Sigma, St Louis, Mo) 6-well plates and incubated in a 5% CO_2 incubator at 37°C.

Trypan Blue Exclusion Assay

Isolated MNCs were cultured for 5 days in EGM-2MV, which was then changed to EBM-2 (Clonetics) with FBS 5%. EPCs were treated with PPAR-δ agonists (GW501516 [Alexis], L-165041 [Sigma]) or vehicle for the whole culture period. At day 8, EPCs were harvested. Cells suspensions were mixed with the same volume of trypan blue, and the numbers of stained and unstained cells were counted over 3 randomly selected high-power fields.

Fluorescence-Activated Cell Sorter Analysis

Cell-cycle status and apoptosis were evaluated by flow cytometry, as described previously.[13] For cell-cycle progression analysis, isolated MNCs were cultured for 5 days in EGM-2MV with PPAR-δ agonists or vehicle. Then adherent cells were harvested.

For analysis of apoptosis, isolated MNCs were cultured in EGM-2MV for 8 days, and the cultured EPCs were treated with PPAR-δ agonists or vehicle in EBM-2 containing FBS 5% for 10 hours. Treated EPCs were subjected to hypoxia (an oxygen concentration of <2%) for 24 hours with the use of the GasPak pouch system (Becton-Dickinson) and then were harvested.

Transendothelial Migration Assay

EPCs were pretreated with PPAR-δ agonists or vehicle under EBM-2 plus FBS 5% for 48 hours. Human gastroepiploic artery endothelial cells were grown on 8-μm microporous membranes in transwell chambers. EPCs labeled with carboxyfluorescein succinimidyl ester (CFSE) (Sigma) (1×10^5/well) were added above GEAEC monolayers on the filters. Then the transwells were placed into a 24-well plate containing EGM-2MV as chemoattractant. After 6 hours of incubation, CFSE-tagged EPCs were counted over 3 randomly selected high-power fields in lower chambers.

Matrigel Tube Formation Assay

EPCs were pretreated with PPAR-δ agonists or vehicle under EBM-2 plus FBS 5% for 48 hours. Growth factor–reduced Matrigel (Becton-Dickinson) was thawed and placed in chamber slides. EPCs (1×10^5) were coplated with (2×10^4) human umbilical vein ECs and incubated at 37°C for 12 hours. Four representative fields were taken, and the averages of the total areas and lengths of complete tubes per unit area were compared with the use of Image-Pro Plus.

Statistical Analysis

All data are presented as mean±SEM. Student t test or 1-way ANOVA was performed for intergroup comparisons. Data taken at different time points were analyzed by repeated-measures ANOVA. SPSS version 12.0 was used for analysis, and a P value of <0.05 was considered statistically significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

PPAR-δ Agonist Induces Akt Phosphorylation in Human EPCs

The signaling pathway underlying PPAR-δ activation in EPCs was investigated by Western blot analysis. We focused on the PI3K/Akt pathway because PPAR-δ activation was shown to be associated with Akt phosphorylation in other cell types.[14,15] In EPCs, the addition of GW501516 (1 μmol/L), a potent and highly selective PPAR-δ agonist, led to a significant increase in serine 473 Akt phosphorylation, reaching a peak at ≈8 hours (Figure 1A) while having no effects on total Akt.

GW501516 Activates PI3K/Akt Signaling via Genomic and Nongenomic Pathways

Akt Activation by GW501516 Is PPAR-δ Dependent

The so-called PPAR-γ agonists are also known to exert PPAR-γ–independent effects.[16,17] To test whether the activation of Akt by GW501516 is dependent on PPAR-δ, PPAR-δ sense and antisense oligodeoxynucleotides were transfected into EPCs with the use of Oligofectamine. The PPAR-δ antisense oligodeoxynucleotide transfection almost completely blocked PPAR-δ mRNA and protein expression specifically (Figure 1B). When PPAR-δ antisense oligodeoxynucleotide was transfected, Akt phosphorylation after 8 hours of GW501516 (1 μmol/L) treatment was not observed (Figure
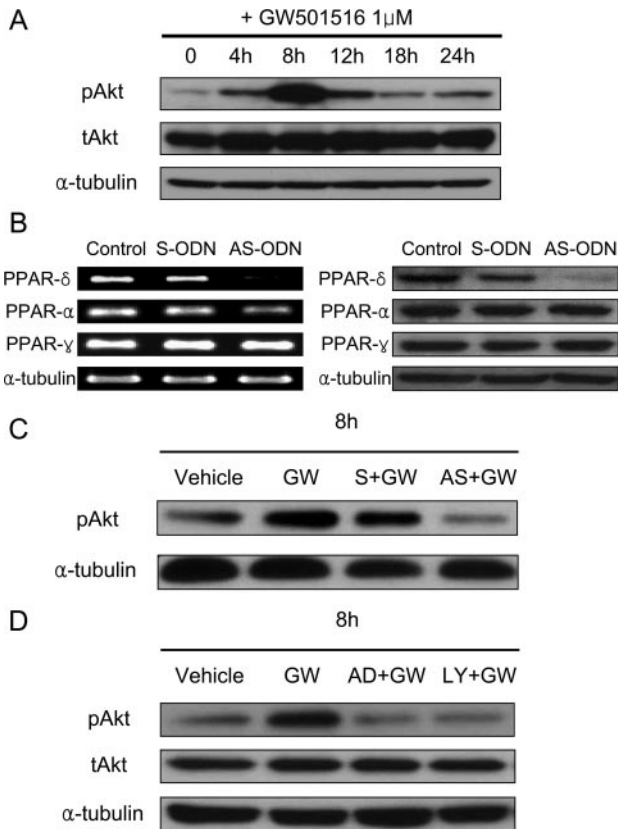
Figure 1. Akt phosphorylation by PPAR-δ agonist in EPCs, mediated via PPAR-δ–dependent genomic transcription. A, The phosphorylation of Akt after GW501516 (1 μmol/L) treatment. B, Specifically downregulated PPAR-δ mRNA and protein expression in EPCs transfected with PPAR-δ antisense (AS) oligodeoxynucleotide (ODN). C, Abolished Akt phosphorylation by PPAR-δ antisense oligodeoxynucleotide transfection. GW indicates GW501516. D, Abolished Akt phosphorylation by pretreatment of actinomycin D (AD) (10 μg/mL) or LY-294002 (LY) (1 μmol/L). All immunoblot assays were performed in triplicate, and each representative result is shown. S indicates sense.
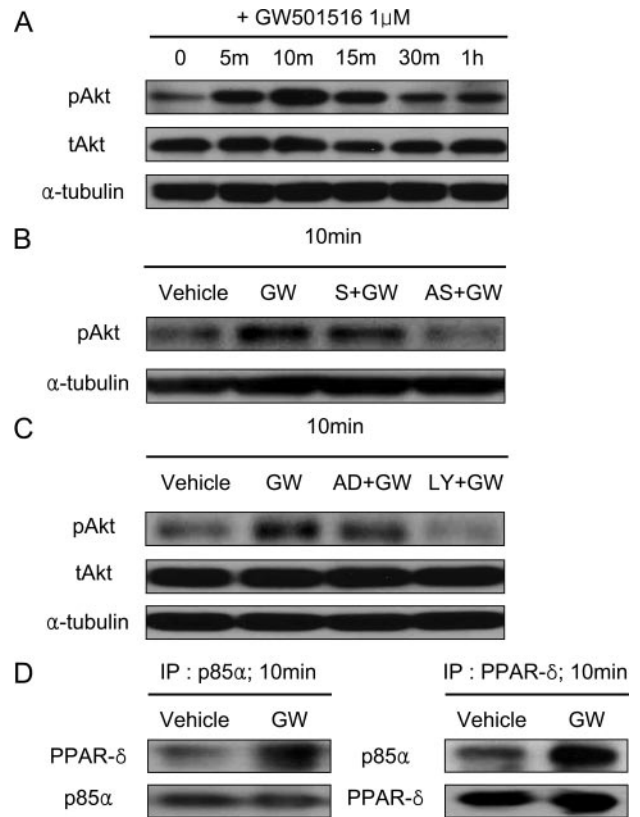
Figure 2. Early Akt phosphorylation by PPAR-δ agonist in EPCs, mediated via PPAR-δ–dependent nongenomic pathway. A, Akt phosphorylation within minutes by GW501516 (1 μmol/L) treatment. B, Abolished Akt phosphorylation after 10 minutes of GW501516 (GW) treatment by PPAR-δ antisense (AS) oligodeoxynucleotide transfection. S indicates sense. C, Preserved Akt phosphorylation after 10 minutes of GW501516 treatment despite actinomycin D (AD) (10 μg/mL) pretreatment. D, Immunoprecipitation (IP) with anti-p85α antibody (or anti-PPAR-δ antibody) and then immunoblotting for PPAR-δ (or p85α). All immunoblot assays and immunoprecipitation assays were performed in triplicate, and each representative result is shown.

1C), suggesting that the activation of Akt by GW501516 occurs through a PPAR-δ–dependent pathway.

Akt Activation by GW501516 Is Mediated via Genomic Transcription

PPAR-δ is a transcription factor itself. Pretreatment of EPCs with actinomycin D, a transcription inhibitor, for 1 hour abolished Akt phosphorylation after 8 hours of GW501516 (1 μmol/L) treatment, suggesting that Akt phosphorylation by PPAR-δ activation occurs via gene transcription. LY-294002, a PI3K inhibitor, pretreatment for 1 hour also diminished Akt phosphorylation, demonstrating that PPAR-δ activation phosphorylates Akt through PI3K (Figure 1D). Neither LY-294002 nor actinomycin D treatment affected EPC viability in the usual culture condition as measured by the trypan blue exclusion assay (data not shown).

GW501516 Also Activates PI3K/Akt via a Nongenomic Pathway Through Interaction With a Regulatory Subunit of PI3K

Previous reports have suggested that mechanisms of nuclear receptor interaction exist at different levels of the signaling pathway, including receptor associations with several transcription factors or other components of the signaling system located at the cell membrane.[18] Interestingly, in pilot experiments to detect the time-dependent changes in Akt phosphorylation by GW501516, we noted that the early peak of Akt phosphorylation at serine 473 had occurred as early as <1 hour after GW501516 treatment (Figure 2A). The phosphorylation of Akt during the early period began within minutes and peaked at 10 minutes. The early phosphorylation of Akt also appeared to be PPAR-δ dependent because PPAR-δ antisense oligodeoxynucleotide transfection blocked Akt phosphorylation after 10 minutes of GW501516 (1 μmol/L) treatment (Figure 2B). However, this early change in Akt phosphorylation did not seem to be a genomic effect of GW501516 because a change within 10 minutes is too early to be mediated by gene transcription and because the early phosphorylation was preserved despite actinomycin D pretreatment. However, LY-294002 abolished Akt phosphorylation after 10 minutes of GW501516 treatment (1 μmol/L) (Figure 2C). Taken together, the aforementioned data suggest

that Akt activation within 10 minutes after GW501516 treatment is a nongenomic effect mediated via PI3K.

Because a previous report showed that nuclear hormone receptors can directly bind to a regulatory subunit to activate PI3K,[19,20] we tested whether the PPAR-δ receptor also directly interacts with p85α, a regulatory subunit of PI3K. An immunoprecipitation assay showed binding of PPAR-δ to the p85α subunit of PI3K in a ligand-dependent manner, suggesting that the early peak in Akt activation may be due to a nongenomic direct interaction between PPAR-δ and p85α (Figure 2D).

These activation mechanisms of the PI3K/Akt pathway by PPAR-δ are depicted in Figure I in the online-only Data Supplement.

PPAR-δ Agonist Increases the Number of EPCs and Protects EPCs From Hypoxia-Induced Apoptosis

The activation of the PI3K/Akt signaling pathway is known to promote EC survival[21] and angiogenesis.[22] In addition, we and others have demonstrated that it is also associated with EPC mobilization and vasculogenesis.[23–25] Thus, the potential of PPAR-δ activation to modulate EPC biology was evaluated.

Trypan blue exclusion assays were performed to quantify the effect of GW501516 on the number of viable EPCs. MNCs were incubated with various concentrations of GW501516 for 8 days. At these concentrations, GW501516 is known to selectively activate only the PPAR-δ receptor with 1000-fold selectivity over the other PPAR subtypes[26,27] and not to affect any other nuclear or nonnuclear receptors.[26] In addition, under oral administration, the serum concentration of GW501516 can reach up to 1 μmol/L in monkeys.[26] GW501516 significantly increased the number of viable EPCs dose dependently, which was reversed by LY-294002 (1 μmol/L) (relative percentage to vehicle [100%]: 118.1±3.2%, 124.0±8.8%, 138.7±12.5%, and 115±6.4% for GW501516 100 nmol/L, 500 nmol/L, 1 μmol/L, and 1 μmol/L+LY-294002, respectively) (Figure 3A, 3B).

To evaluate whether this increase of cell number was due to proliferation, the cell cycle was analyzed in 5-day cultured EPCs that had been incubated under GW501516 after MNC isolation. GW501516 significantly increased the number of EPCs in the S phase, which was reversed again by LY-294002 pretreatment (relative percentage to vehicle [100%]: 123.5±18.7%, 150.5±15.3%, 171.2±19.2%, and 122.6± 13.7% for GW501516 100 nmol/L, 500 nmol/L, 1 μmol/L, and 1 μmol/L+LY-294002, respectively) (Figure 3C).

Next, the effect of PPAR-δ activation on apoptosis of EPCs was tested. EPCs were treated with GW501516 and then exposed to hypoxia. After 24 hours, the subdiploid cell fraction, indicative of cells with DNA fragmentation during apoptosis, was decreased in cells treated with GW501516, which was reversed by LY-294002 (percentage of apoptotic cells: 23.6±2.0% versus 19.6±2.0%, 18.5±1.5%, 10.9± 0.6%, and 27.8±2.0% for vehicle versus GW501516 100 nmol/L, 500 nmol/L, 1 μmol/L, and 1 μmol/L+LY-294002, respectively), suggesting protection of EPCs from hypoxia-induced apoptosis by GW501516 (Figure 3D, 3E).

Another PPAR-δ agonist, L-165041, which has been used to activate PPAR-δ in several studies,[14,28] also induced proliferation of EPCs and protected them from apoptosis (Figure IIA to IIE in the online-only Data Supplement).

PPAR–δ Agonist Enhances EPC Function

The effects of PPAR-δ activation on EPC function were assessed by measuring transendothelial migration and in vitro tube formation. CFSE-tagged EPCs treated with GW501516 for 48 hours were placed on a chamber on top of a microporous membrane layered with human gastroepiploic artery endothelial cells, and 6 hours later, the number of CFSE-tagged EPCs that migrated to the lower chamber was counted. GW501516 increased transendothelial migration of EPCs (relative percentage to vehicle [100%]: 110.9±13.3%, 128.9±11.2%, 151.6± 13.6%, and 101.9±7.5% for GW501516 100 nmol/L, 500 nmol/L, 1 μmol/L, and 1 μmol/L+LY-294002, respectively) (Figure 4A, 4B).

In vitro tube formation by EPCs was measured with the use of a Matrigel network formation assay. Naive human umbilical vein ECs were cocultured on Matrigel with EPCs treated with GW501516. After 12 hours, the area and length of complete tubes were analyzed. GW501516-treated EPCs induced a significantly larger area (relative percentage to vehicle [100%]: 288.3±18.2%, 324.1±47.0%, 320.7± 11.6%, and 147±25.0% for GW501516 100 nmol/L, 500 nmol/L, 1 μmol/L, and 1 μmol/L+LY-294002, respectively) and longer length of complete tubes (relative percentage to vehicle [100%]: 183.8±3.6%, 218.2±10.2%, 238.9±7.2%, and 122±16.2% for GW501516 100 nmol/L, 500 nmol/L, 1 μmol/L, and 1 μmol/L+LY-294002, respectively) (Figure 4C, 4D).

The augmented migration and tube formation by EPCs treated with GW501516 (1 μmol/L) were all reversed by LY-294002 (1 μmol/L) pretreatment (Figure 4A to 4D), suggesting that these effects are mediated via the PI3K/Akt pathway. LY-294002 treatment did not affect EPC viability as measured by the trypan blue exclusion assay (data not shown).

L-165041 also enhanced EPC functions such as transendothelial migration and in vitro tube formation (Figure IIIA to IIID in the online-only Data Supplement), suggesting that these effects are dependent on PPAR-δ activation and not specific to GW501516.

Local Transplantation of PPAR-δ–Activated EPCs Enhances Vasculogenesis in Mouse Ischemic Hindlimb

To assess the impact of PPAR-δ activation on EPCs in vivo, injection of GW501516-treated human EPCs was performed in hindlimb ischemia model of athymic nude mice. After unilateral femoral artery excision, athymic nude mice received either GW501516- or vehicle-treated human EPCs (n=12 in each group) labeled with 1,1′-dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate (DiI) via direct muscular injection. Quantitative analysis with the use of a laser Doppler perfusion imager confirmed significantly accelerated limb perfusion improvement throughout the 21-day
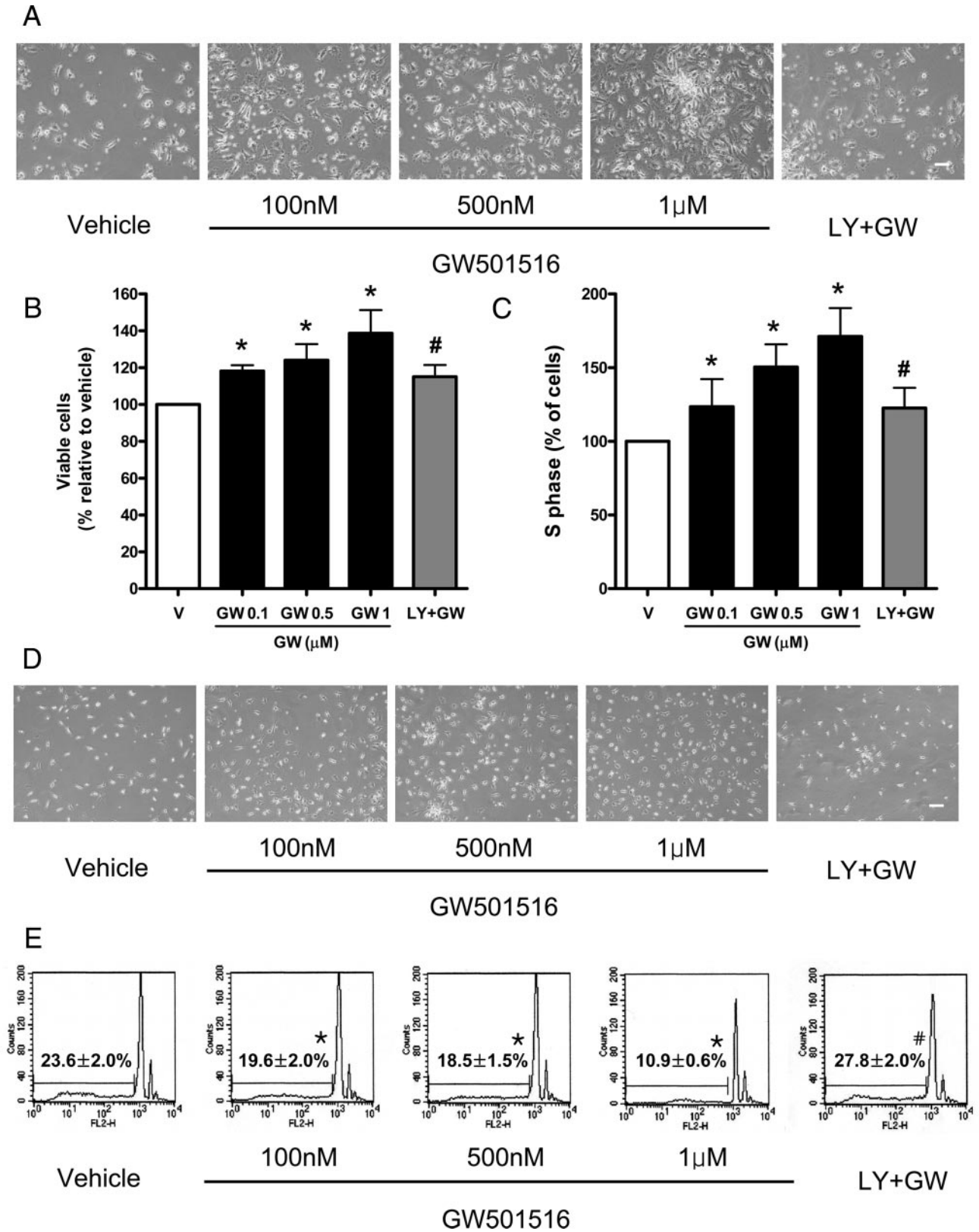
Figure 3. Effects of PPAR-δ activation on proliferation and apoptosis of human EPCs through PI3K/Akt activation. A, Representative figure after 8 days of culture. B, Quantitative graph of the number of viable EPCs. LY indicates LY-294002. C, The number of EPCs in the S-phase in fluorescence-activated cell sorter analysis. D, Representative figure after 24 hours of exposure to hypoxia. E, The sub-diploid fraction of EPCs in fluorescence-activated cell sorter analysis. Data are mean±SEM; n=3. *$P<0.05$ vs vehicle (V), #$P<0.05$ vs GW501516 (GW) (1 μmol/L). Bars=100 μm.
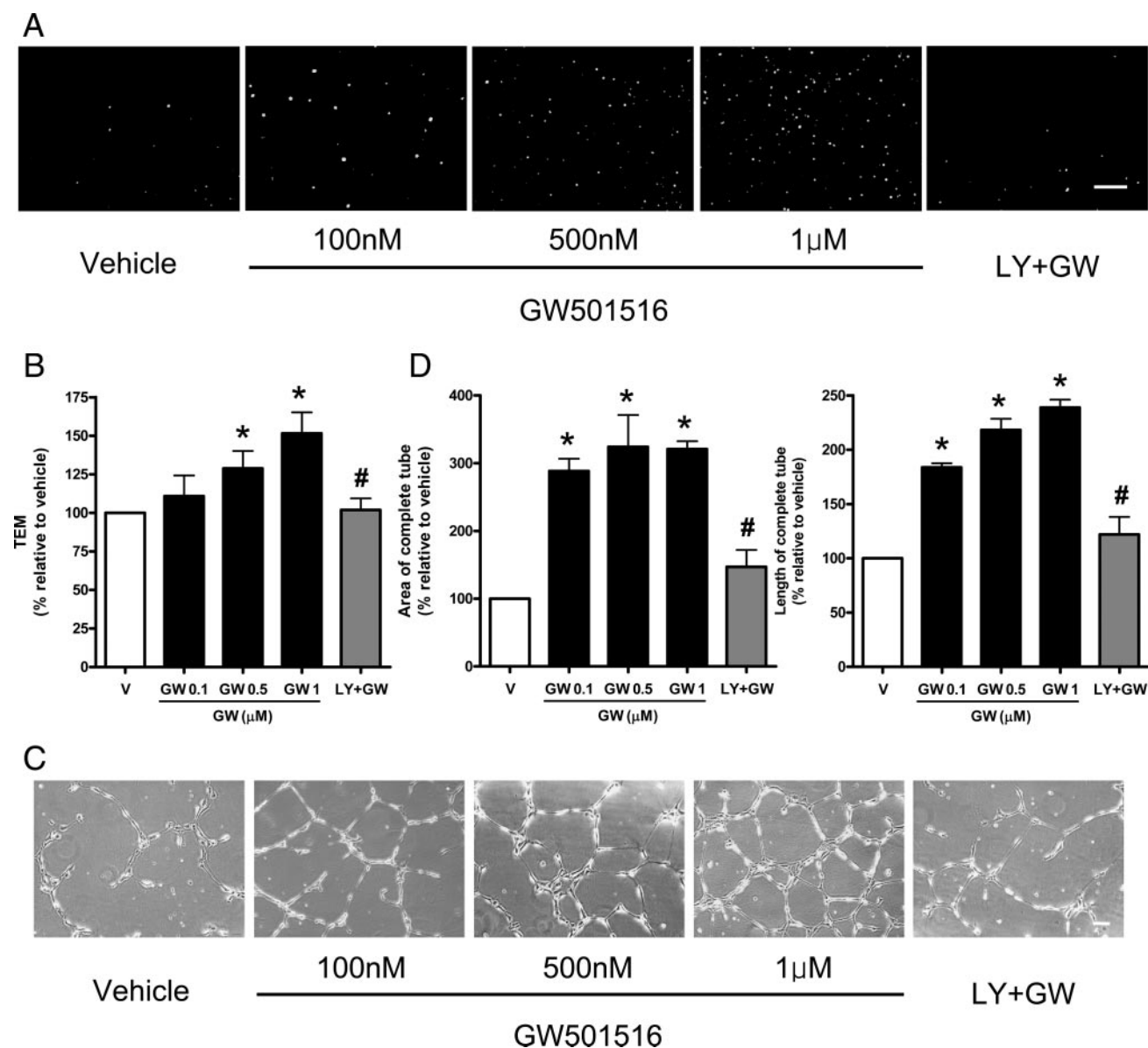
Figure 4. Effects of PPAR-δ activation on various human EPC functions through PI3K/Akt activation. A, Representative figure showing CFSE-tagged EPCs that migrated to the lower chamber. B, Quantitative graph of the number of migrated EPCs. C, Representative figure showing in vitro tube formation. D, Quantitative graph of the area and length of complete tubes. TEM indicates transendothelial migration; LY, LY-294002. Data are mean±SEM; n=3. *$P<0.05$ vs vehicle (V), #$P<0.05$ vs GW501516 (GW) (1 μmol/L). Bars=100 μm.

follow-up period in the hindlimbs injected with the GW501516-treated EPCs (Figure 5A, 5B). Histological examination of skeletal muscle of ischemic limb at day 21 showed significantly increased capillary density in mice transplanted with GW501516-treated EPCs (capillary density, $541.0\pm93.3/mm^2$ versus $302.8\pm35.5/mm^2$ for mice receiving GW501516-treated EPCs versus control EPCs) (Figure 5C, 5D). A greater number of human DiI-labeled EPCs was detected in the ischemic limb of mice given GW501516-treated EPCs (human EPC density, $70.8\pm9.3/mm^2$ versus $47.9\pm4.8/mm^2$ for mice receiving GW501516-treated EPCs versus control EPCs) (Figure 5C, 5E). These data suggest that ex vivo activation of PPAR-δ in EPCs results in enhanced vasculogenic potential in vivo.

To test whether this in vivo provasculogenic effect of GW501516 is dependent on PPAR-δ, PPAR-δ–knockout (KO) mice were used. Mouse EPCs from PPAR-δ KO or wild-type (WT) mice were cultured with GW501516 or vehicle and injected intramuscularly to the ischemic hindlimb. Blood flow recovery was significantly augmented by GW501516 pretreatment of transplanted EPCs from WT mice, whereas it was not from PPAR-δ KO mice (Figure IVA, IVB in the online-only Data Supplement). In histological examination at day 21, higher capillary density and more accumulation of mouse EPCs were observed only in mice receiving GW501516-treated WT mouse EPCs but not in mice receiving GW501516-treated PPAR-δ KO mouse EPCs (Figure IVC to IVE in the online-only Data Supplement),
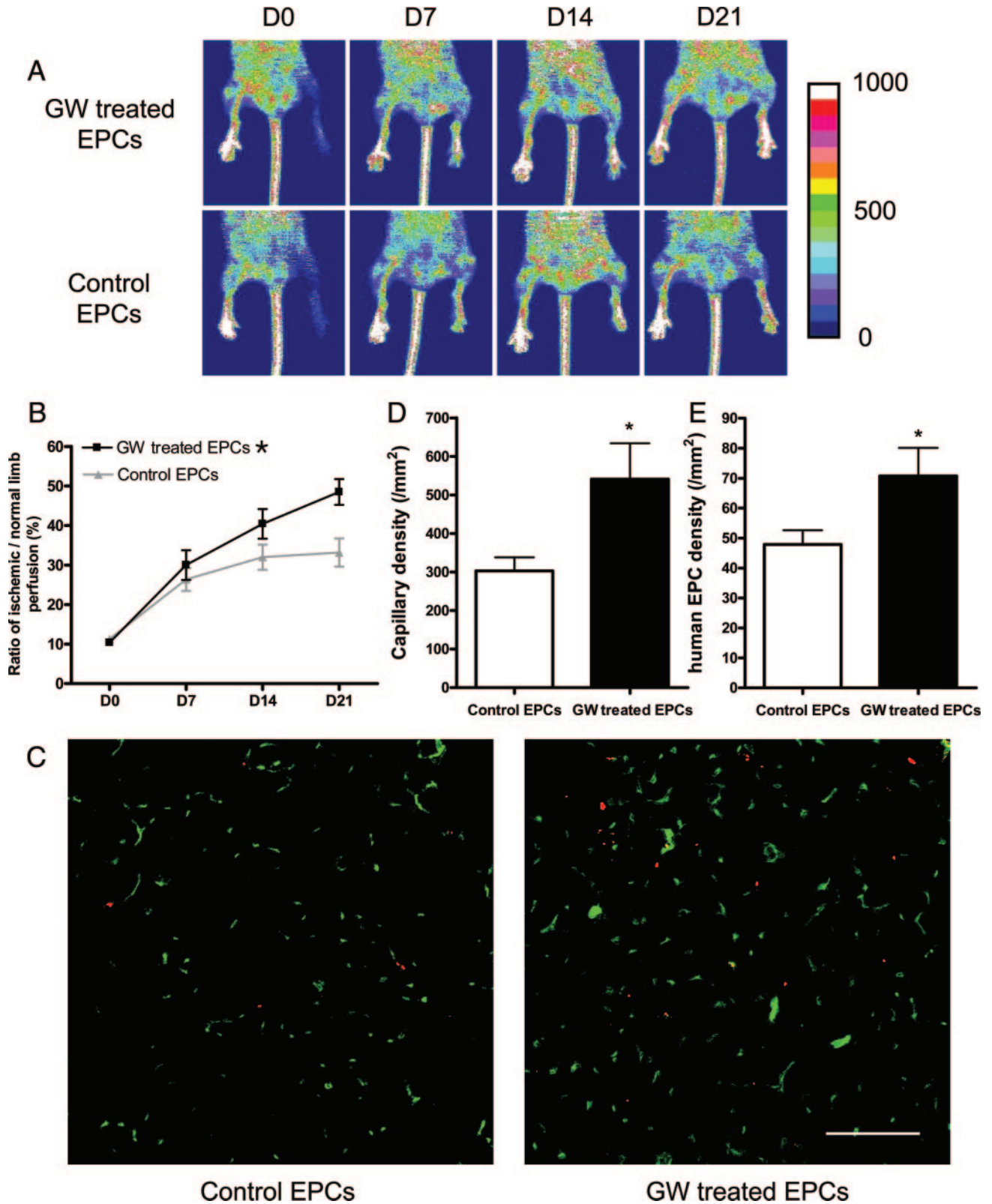
Figure 5. In vivo vasculogenic effects of ex vivo PPAR-δ–activated human EPCs in hindlimb ischemia model of athymic nude mice. A, Representative limb perfusion images obtained by laser Doppler perfusion imager. B, The quantified ratios of the perfusions of ischemic and nonischemic limbs. C, Representative histological findings of ischemic limbs of each group. Human EPCs were labeled with DiI (red) and mouse ECs with BS-1 lectin (green). D, Quantitative analysis of capillary density. E, Quantitative analysis of human EPC density. GW indicates GW501516. Data are mean±SEM; n=12 in each group. *$P<0.05$ vs control. Bar=100 μm.
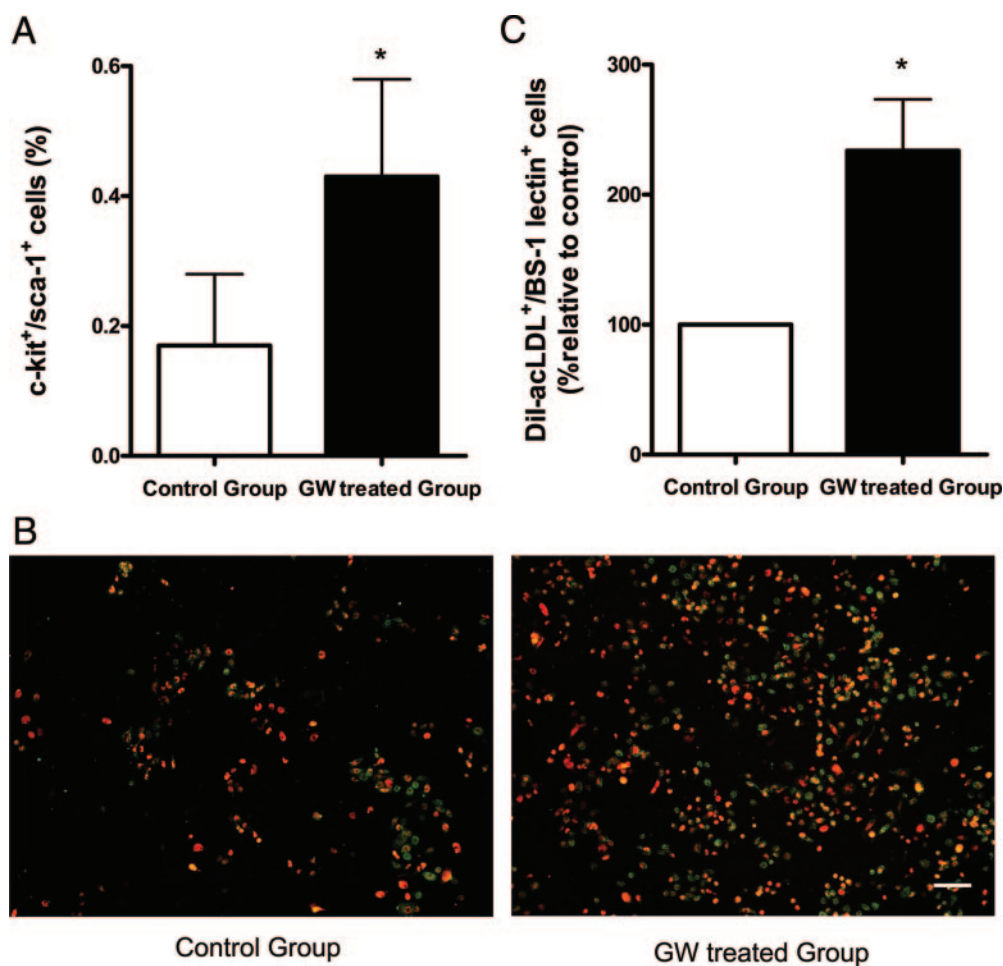
Figure 6. Effects of systemic administration of PPAR-δ agonist on HSCs in the BM and EPCs in the peripheral blood of mice. A, Fluorescence-activated cell sorter analysis of the number of HSCs in BM, as represented by c-kit/sca-1 coexpression. B, Representative figure after 8 days of peripheral MNC culture. Red indicates DiI-acLDL uptake; green, BS-1 lectin binding. Double-positive cells represent EPCs. C, Quantitative graph of the number of EPCs. GW indicates GW501516. Data are mean±SEM; n=6 in each group. *P<0.05 vs control. Bar=100 μm.

suggesting that GW501516 exerts its provasculogenic effect through PPAR-δ.

Systemic Administration of PPAR-δ Agonist Enhances EPC Mobilization and Vasculogenesis at the Injured Cornea of Mice That Received BM Transplantation From Green Fluorescent Protein–Transgenic Mice

To determine whether systemic PPAR-δ activation actually increases the number of EPCs in vivo, we counted EPCs in the peripheral blood as well as multipotent hematopoietic stem cells (HSCs) in BM after systemic administration of GW501516. Mice were treated with GW501516 or vehicle for 14 days (n=6 in each group), and then BM and whole peripheral blood were obtained. The percentage of multipotent HSCs, as represented by the coexpression of c-kit and sca-1, was found to be significantly higher in the BM of the GW501516-treated mice (percentage of c-kit/sca-1 double-positive cells: 0.17±0.11% versus 0.43±0.15% for control group versus GW501516-treated group) (Figure 6A).

After 8 days of peripheral MNC culture, EPCs were confirmed by a combination of DiI-labeled acetylated low-density lipoprotein (DiI-acLDL) uptake and *Bandeiraea simplicifolia* (BS)-1 lectin binding. The number of EPCs from the peripheral blood was significantly higher in the GW501516-treated group (relative percentage to control [100%]: 233.9±39.6% for GW501516-treated group) (Figure 6B, 6C).

To establish whether these EPC mobilization effects of systemic PPAR-δ activation were actually associated with augmented vasculogenesis, the effects of GW501516 therapy on new vessel formation were tested in a murine model of corneal neovascularization after BM transplantation. WT mice received BM transplantation from donor green fluorescent protein (GFP)–transgenic mice. After completing a 14-day course of GW501516 or control vehicle administration, transplanted mice underwent corneal microsurgery (n=5 in each group). The corneas of mice were harvested at 6 days after surgery. During these 6 days, GW501516 or vehicle administration was maintained. GW501516 therapy resulted in enhanced corneal neovascularization (Figure 7A). Quantitative analysis showed that GW501516 increased both the length and number of microvessels from limbal arteries in injured corneas (length, 0.32±0.05 mm in control group,
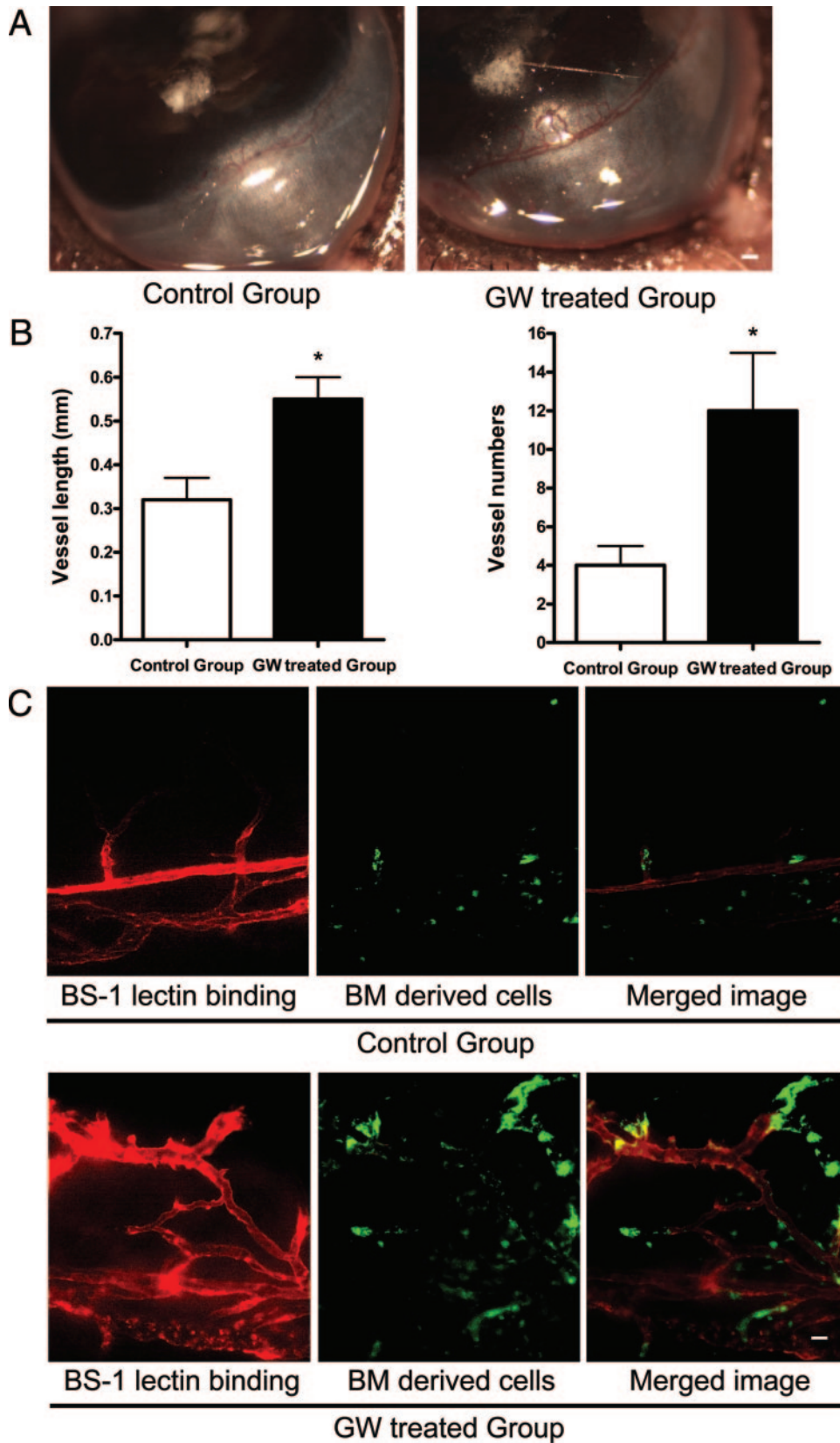
Figure 7. In vivo vasculogenic effects of systemic administration of PPAR-δ agonist in a murine BM transplantation and corneal neovascularization model. A, Representative figure showing enhanced corneal neovascularization. B, Quantitative graph of the lengths and numbers of sprouting microvessels from limbal arteries. C, Representative figure of fluorescence staining of whole mounted corneas showing incorporation of BM-derived cells. Red represents BS-1 lectin binding endothelial cells; green, GFP-positive BM-derived cells. Data are mean±SEM; n=5 in each group. *$P<0.05$ vs control. Bars=200 μm (A); 100 μm (C).

0.55±0.05 mm in GW501516-treated group; number, 4±1 in control group, 12±3 in GW501516-treated group) (Figure 7B). In vivo fluorescence staining of corneas with BS-1 lectin showed greater neovascularization and more extensive incorporation of BM-derived GFP-positive cells in the GW501516 group (Figure 7C).

Systemic Administration of PPAR-δ Agonist Enhances Vasculogenesis in Mouse Ischemic Hindlimb

In the aforementioned experiments, PPAR-δ activation in EPCs before transplantation resulted in the enhancement of vasculogenic potential of EPCs for ischemic limb. Then we assessed the effects of systemic PPAR-δ activation on the vasculogenesis in the mouse ischemic hindlimb model. After a 3-day course of intraperitoneal administration of GW501516 or vehicle, C57BL/6 mice underwent unilateral femoral artery excision (n=10 in each group). GW501516 or vehicle therapy was maintained for 21 days after surgery. Serial follow-up with the use of a laser Doppler perfusion imager showed better blood flow to the ischemic limb in the mice injected with GW501516 (Figure 8A, 8B). At day 21 after surgery with systemic PPAR-δ activation, capillary density of ischemic limb was significantly higher in the GW501516-treated mice (capillary density, 795.9±149.7/ mm^2 versus 452.4±97.9/mm^2 for GW501516-treated group versus control group) (Figure 8C, 8D). Confocal microscopic examination of CD31-immunofluorescent staining of thick cryosections demonstrated a robust vasculogenesis in the ischemic limb of GW501516-treated mice (Figure 8E).

Discussion

Since Asahara and colleagues[1] reported the presence of EPCs in the adult peripheral blood in 1997, many researchers have focused interest on EPC biology and its clinical application. Although the therapeutic potential of EPCs in treating ischemic vascular disease seems promising, its application is hindered by the limited number of available EPCs. Thus, efforts to modulate EPCs with drugs[24,25] or by genetic modifications[29,30] are under intense study.

PPAR-δ is expressed in diverse tissues and cells,[3,7,26] but its function outside the metabolic effects is poorly understood. Its reported roles in vascular biology include the induction of EC proliferation,[8,9] angiogenesis,[9] and EC protection from H$_2$O$_2$-induced apoptosis.[10]

Our experiments provide the first evidence that PPAR-δ activation has a provasculogenic effect both in vitro and in vivo. We found that PPAR-δ activation increased the proliferation of EPCs and protected them from apoptosis. Moreover, PPAR-δ activation significantly enhanced EPC functions. Then the in vivo provasculogenic effects of PPAR-δ activation were confirmed in 2 different aspects. First, in the mouse ischemic hindlimb model, we showed that local delivery of ex vivo PPAR-δ–activated EPCs significantly enhanced new vessel formation, resulting in better limb perfusion. The PPAR-δ specificity of these beneficial effects was confirmed by PPAR-δ KO mice. These results suggest that the clinical impact of local delivery of EPCs modulated with PPAR-δ agonist is great compared with naive EPCs that have been used in clinical trials. Second, the systemic effects of PPAR-δ agonist were also studied. Systemic administration of PPAR-δ agonist significantly increased the number of HSCs in BM, leading to EPC mobilization. These effects resulted in improved vasculogenesis in a corneal neovascularization model and limb salvage with better blood flow in an ischemic hindlimb model. These results indicate that the systemic administration of PPAR-δ agonist by itself has therapeutic potential.

Other interesting and novel findings of the present study are that the beneficial effects observed with PPAR-δ activation in EPCs are mediated by the PI3K/Akt pathway, a key pathway that mediates vasculogenesis.[23–25] In our study, GW501516 also activated the PI3K/Akt pathway, suggesting that PPAR-δ agonist may be yet another drug that can target the activation of the PI3K/Akt signaling pathway in EPCs. The activation of the PI3K/Akt pathway by PPAR-δ activation has been reported previously in other cell types such as keratinocytes[14] and lung cancer cell lines,[15] but this is a novel finding in EPCs. In keratinocytes, PPAR-δ modulates Akt phosphorylation via transcriptional upregulation of integrin-linked kinase.[14] However, in our Western blot assay, there was no change in integrin-linked kinase up to 24 hours after GW501516 treatment (data not shown). The specificity and potency of activation of genomic target genes by PPARs are known to be highly dependent on the cell type,[31] which might explain the observed difference in integrin-linked kinase production.

We also first showed that PPAR-δ activation led to the phosphorylation of Akt via not only the genomic pathway but also the nongenomic pathway. We found another peak of Akt phosphorylation as early as 10 minutes after PPAR-δ activation, and this could not be inhibited by actinomycin D treatment, suggesting that such an effect is not dependent on transcription and is thus a nongenomic effect. Glucocorticoids and estrogen are known to nontranscriptionally activate the PI3K/Akt pathway in ECs.[19,20] This activation arises within minutes and is not blocked by actinomycin D. The nontranscriptional activation in cytoplasm was shown to be a result of interaction in the cytoplasm between the glucocorticoid receptor or estrogen receptor and p85α, a regulatory subunit of PI3K.[19,20] Glucocorticoid receptor and estrogen receptor are members of the nuclear hormone receptor superfamily similar to PPAR-δ, and these receptors have a conserved domain structure.[32] Therefore, we hypothesized that PPAR-δ might activate PI3K nontranscriptionally by the same mechanism. Indeed, we showed by immunoprecipitation that the agonist increased PPAR-δ binding with p85α in the early period.

Several effects of "PPAR-γ agonists," the most extensively studied PPAR subtype, have been shown to be PPAR-γ independent.[16,17] However, in the present study, 2 different PPAR-δ agonists (ie, GW501516, L-165041) exerted the same in vitro effects on EPCs, confirming that these effects are PPAR-δ dependent. Furthermore, EPCs from PPAR-δ KO mice did not show in vivo provasculogenic effects after GW501516 treatment in contrast to those from WT mice. Moreover, the genomic and nongenomic activation of the PI3K/Akt pathway by GW501516 was dependent on
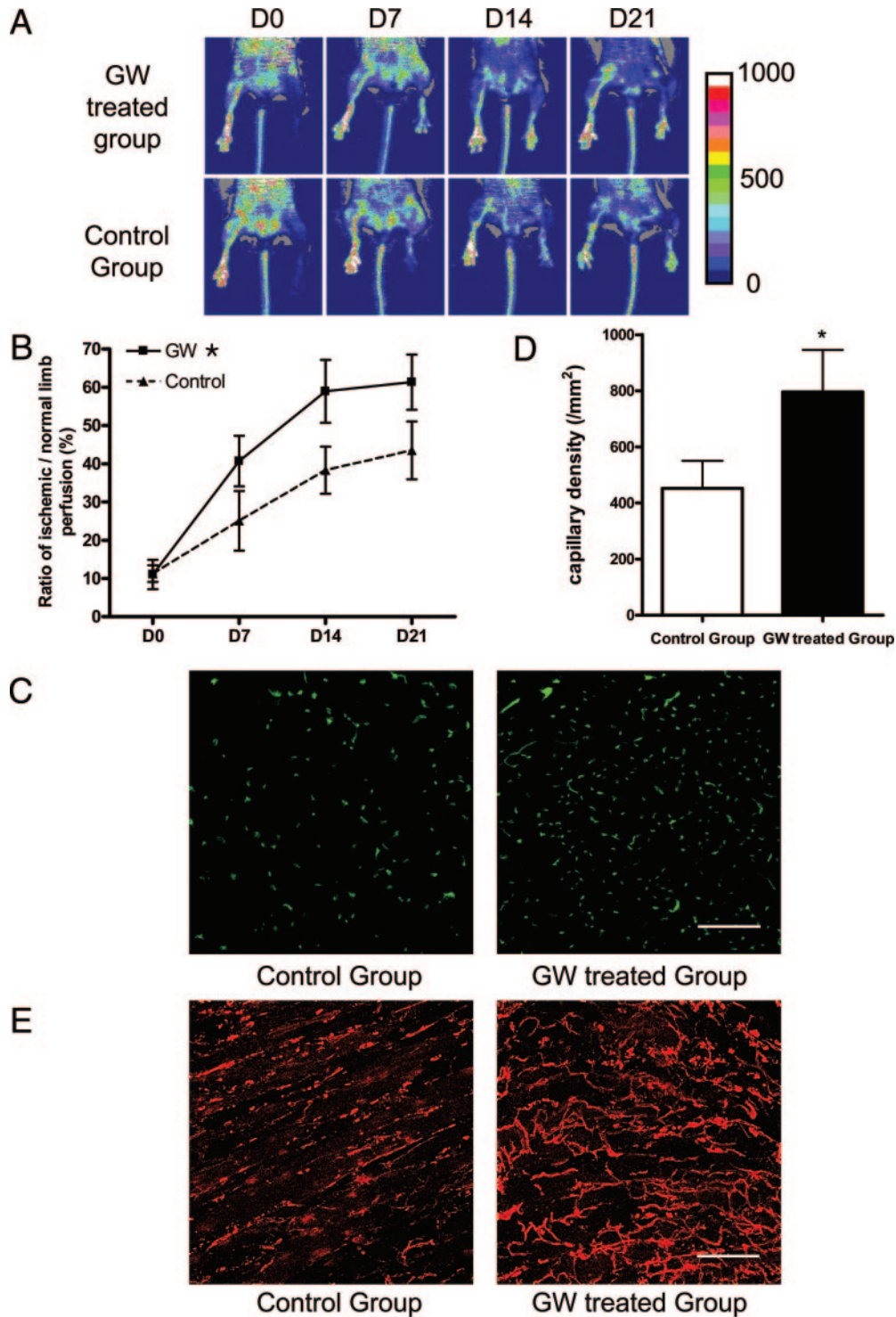
Figure 8. The effects of systemic administration of PPAR-δ agonist in hindlimb ischemia model of mice. A, Representative limb perfusion images obtained by laser Doppler perfusion imager. B, The quantified ratios of the perfusions of ischemic and nonischemic limbs. C, Representative histological findings of ischemic limbs of each group. ECs were labeled with BS-1 lectin (green). D, Quantitative analysis of capillary density. E, Representative pictures of thick section of ischemic limbs. ECs were labeled with CD31 (red). GW indicates GW501516. Data are mean±SEM; n=10 in each group. *$P<0.05$ vs control. Bars=100 μm (C); 50 μm (E).

PPAR-δ, as verified by an antisense oligodeoxynucleotide transfection assay. Therefore, we concluded that the results in this study are dependent on PPAR-δ.

Three different processes of vascular growth exist. Vasculogenesis describes the formation of blood vessels from EPCs. Angiogenesis denotes the sprouting of capillaries from preexisting ECs. The growth and remodeling of a collateral artery is termed *arteriogenesis*.[33,34] In an ischemic hindlimb model, arteriogenesis is known to occur mainly in the upper part of the limb; on the contrary, angiogenesis and vasculogenesis are driven by tissue ischemia in the lower part.[34] The greater vascular incorporation of BM-derived cells by

PPAR-δ agonist in a corneal injury model suggests that vasculogenesis may at least work in perfusion improvement by PPAR-δ agonist in our hindlimb ischemia models. EPCs may also induce angiogenesis and arteriogenesis in ischemic limb through a paracrine effect.[35] The discussion of the exact proportion of contributions from these different processes to the recovery of limb perfusion is not within the scope of this study and may require further study.

In conclusion, our data suggest that PPAR-δ agonist enhances vasculogenesis and may be a novel therapeutic option to modulate EPCs in the treatment of ischemic vascular diseases.

Sources of Funding

Disclosures

None.

References

1. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. *Science*. 1997;275:964–967.
2. Vasa M, Fichtlscherer S, Adler K, Aicher A, Martin H, Zeiher AM, Dimmeler S. Increase in circulating endothelial progenitor cells by statin therapy in patients with stable coronary artery disease. *Circulation*. 2001; 103:2885–2890.
3. Berger J, Moller DE. The mechanisms of action of PPARs. *Annu Rev Med*. 2002;53:409–435.
4. Margeli A, Kouraklis G, Theocharis S. Peroxisome proliferator–activated receptor-γ ligands and angiogenesis. *Angiogenesis*. 2003;6:165–169.
5. Verma S, Kuliszewski MA, Li SH, Szmitko PE, Zucco L, Wang CH, Badiwala MV, Mickle DA, Weisel RD, Fedak PW, Stewart DJ, Kutryk MJ. C-reactive protein attenuates endothelial progenitor cell survival, differentiation, and function. *Circulation*. 2004;109:2058–2067.
6. Panigrahy D, Singer S, Shen LQ, Butterfield CE, Freedman DA, Chen EJ, Moses MA, Kilroy S, Duensing S, Fletcher C, Fletcher JA, Hlatky L, Hahnfeldt P, Folkman J, Kaipainen A. PPAR-γ ligands inhibit primary tumor growth and metastasis by inhibiting angiogenesis. *J Clin Invest*. 2002;110:923–932.
7. Berger JP, Akiyama TE, Meinke PT. PPARs: therapeutic targets for metabolic disease. *Trends Pharmacol Sci*. 2005;26:244–251.
8. Stephen RL, Gustafsson MC, Jarvis M, Tatoud R, Marshall BR, Knight D, Ehrenborg E, Harris AL, Wolf CR, Palmer CN. Activation of peroxisome proliferator–activated receptor-δ stimulates the proliferation of human breast and prostate cancer cell lines. *Cancer Res*. 2004;64: 3162–3170.
9. Piqueras L, Reynolds AR, Hodivala-Dilke KM, Alfranca A, Redondo JM, Hatae T, Tanabe T, Warner TD, Bishop-Bailey D. Activation of PPARbeta/delta induces endothelial cell proliferation and angiogenesis. *Arterioscler Thromb Vasc Biol*. 2007;27:63–69.
10. Liou JY, Lee S, Ghelani D, Matijevic-Aleksic N, Wu KK. Protection of endothelial survival by peroxisome proliferator–activated receptor-δ mediated 14-3-3 upregulation. *Arterioscler Thromb Vasc Biol*. 2006;26: 1481–1487.
11. Hur J, Yoon CH, Kim HS, Choi JH, Kang HJ, Hwang KK, Oh BH, Lee MM, Park YB. Characterization of two types of endothelial progenitor cells and their different contributions to neovasculogenesis. *Arterioscler Thromb Vasc Biol*. 2004;24:288–293.
12. Yoon CH, Hur J, Park KW, Kim JH, Lee CS, Oh IY, Kim TY, Cho HJ, Kang HJ, Chae IH, Yang HK, Oh BH, Park YB, Kim HS. Synergistic neovascularization by mixed transplantation of early endothelial progenitor cells and late outgrowth endothelial cells: the role of angiogenic cytokines and matrix metalloproteinases. *Circulation*. 2005;112: 1618–1627.
13. Kim HS, Skurk C, Thomas SR, Bialik A, Suhara T, Kureishi Y, Birnbaum M, Keaney JF Jr, Walsh K. Regulation of angiogenesis by glycogen synthase kinase-3beta. *J Biol Chem*. 2002;277:41888–41896.
14. Di-Poi N, Tan NS, Michalik L, Wahli W, Desvergne B. Antiapoptotic role of PPARβ in keratinocytes via transcriptional control of the Akt1 signaling pathway. *Mol Cell*. 2002;10:721–733.
15. Han S, Ritzenthaler JD, Wingerd B, Roman J. Activation of PPAR β/δ increases the expression of prostaglandin E2 receptor subtype EP4. *J Biol Chem*. 2005;280:33240–33249.
16. Hinz B, Brune K, Pahl A. 15-Deoxy-Δ12,14-prostaglandin J2 inhibits the expression of proinflammatory genes in human blood monocytes via a PPAR-γ independent mechanism. *Biochem Biophys Res Commun*. 2003; 302:415–420.
17. Okura T, Nakamura M, Takata Y, Watanabe S, Kitami Y, Hiwada K. Troglitazone induces apoptosis via the p53 and Gadd45 pathway in vascular smooth muscle cells. *Eur J Pharmacol*. 2000;407:227–235.
18. Bonofiglio D, Gabriele S, Aquila S, Catalano S, Gentile M, Middea E, Giordano F, Ando S. Estrogen receptor α binds to peroxisome proliferator–activated receptor response element and negatively interferes with peroxisome proliferator–activated receptor γ signaling in breast cancer cells. *Clin Cancer Res*. 2005;11:6139–6147.
19. Simoncini T, Hafezi-Moghadam A, Brazil DP, Ley K, Chin WW, Liao JK. Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. *Nature*. 2000;407:538–541.
20. Hafezi-Moghadam A, Simoncini T, Yang Z, Limbourg FP, Plumier JC, Rebsamen MC, Hsieh CM, Chui DS, Thomas KL, Prorock AJ, Laubach VE, Moskowitz MA, French BA, Ley K, Liao JK. Acute cardiovascular protective effects of corticosteroids are mediated by non-transcriptional activation of endothelial nitric oxide synthase. *Nat Med*. 2002;8:473–479.
21. Fujio Y, Walsh K. Akt mediates cytoprotection of endothelial cells by vascular endothelial growth factor in an anchorage-dependent manner. *J Biol Chem*. 1999;274:16349–16354.
22. Kureishi Y, Luo Z, Shiojima I, Bialik A, Fulton D, Lefer DJ, Sessa WC, Walsh K. The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals. *Nat Med*. 2000;6:1004–1010.
23. Hur J, Yoon CH, Lee CS, Kim TY, Oh IY, Park KW, Kim JH, Lee HS, Kang HJ, Chae IH, Oh BH, Park YB, Kim HS. Akt is a key modulator of endothelial progenitor cell trafficking in ischemic muscle. *Stem Cells*. 2007;25:1769–1778.
24. Dimmeler S, Aicher A, Vasa M, Mildner-Rihm C, Adler K, Tiemann M, Rutten H, Fichtlscherer S, Martin H, Zeiher AM. HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway. *J Clin Invest*. 2001;108:391–397.
25. Llevadot J, Murasawa S, Kureishi Y, Uchida S, Masuda H, Kawamoto A, Walsh K, Isner JM, Asahara T. HMG-CoA reductase inhibitor mobilizes bone marrow-derived endothelial progenitor cells. *J Clin Invest*. 2001; 108:399–405.
26. Oliver WR Jr, Shenk JL, Snaith MR, Russell CS, Plunket KD, Bodkin NL, Lewis MC, Winegar DA, Sznaidman ML, Lambert MH, Xu HE, Sternbach DD, Kliewer SA, Hansen BC, Willson TM. A selective peroxisome proliferator–activated receptor delta agonist promotes reverse cholesterol transport. *Proc Natl Acad Sci U S A*. 2001;98:5306–5311.
27. 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 Jr, Sternbach DD. Novel selective small molecule agonists for peroxisome proliferator–activated receptor delta (PPARdelta)-synthesis and biological activity. *Bioorg Med Chem Lett*. 2003;13:1517–1521.
28. Kim DJ, Akiyama TE, Harman FS, Burns AM, Shan W, Ward JM, Kennett MJ, Gonzalez FJ, Peters JM. Peroxisome proliferator–activated receptor β (δ)-dependent regulation of ubiquitin C expression contributes to attenuation of skin carcinogenesis. *J Biol Chem*. 2004;279: 23719–23727.
29. Choi JH, Hur J, Yoon CH, Kim JH, Lee CS, Youn SW, Oh IY, Skurk C, Murohara T, Park YB, Walsh K, Kim HS. Augmentation therapeutic angiogenesis using genetically modified human endothelial progenitor cells with altered glycogen synthase kinase-3β activity. *J Biol Chem*. 2004;279:49430–49438.
30. Cho HJ, Youn SW, Cheon SI, Kim TY, Hur J, Zhang SY, Lee SP, Park KW, Lee MM, Choi YS, Park YB, Kim HS. Regulation of endothelial cell and endothelial progenitor cell survival and vasculogenesis by integrin-linked kinase. *Arterioscler Thromb Vasc Biol*. 2005;25:1154–1160.
31. Nielsen R, Grøntved L, Stunnenberg HG, Mandrup S. Peroxisome proliferator–activated receptor subtype- and cell-type-specific activation of

genomic target genes upon adenoviral transgene delivery. *Mol Cell Biol*. 2006;26:5698–5714.

32. Huss JM, Kelly DP. Nuclear receptor signaling and cardiac energetics. *Circ Res*. 2004;95:568–578.

33. Semenza GL. Vasculogenesis, angiogenesis, and arteriogenesis: mechanisms of blood vessel formation and remodeling. *J Cell Biochem*. 2007; 102:840–847.

34. Mees B, Wagner S, Ninci E, Tribulova S, Martin S, van Haperen R, Kostin S, Heil M, de Crom R, Schaper W. Endothelial nitric oxide synthase activity is essential for vasodilation during blood flow recovery but not for arteriogenesis. *Arterioscler Thromb Vasc Biol*. 2007;27:1926–1933.

35. Rehman J, Li J, Orschell CM, March KL. Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors. *Circulation*. 2003;107:1164–1169.

CLINICAL PERSPECTIVE

Since the early 2000s, peroxisome proliferator–activated receptors (PPARs), especially PPAR-γ, have been under intense study in various areas, including vascular biology. Although PPAR-γ agonist was found to exert several adverse effects such as weight gain and low-density lipoprotein elevation, it was also known to have many beneficial effects on conditions such as atherosclerosis and postangioplasty restenosis, and the commercial ligands have been prescribed widely as an antidiabetic drug. However, recent studies raised important questions about the cardiovascular safety of PPAR-γ agonist. Naturally, other PPARs have awakened researchers' interest as an alternative choice. The function of PPAR-δ remained mainly unexplored until recently. In our study, PPAR-δ activation was revealed to regulate endothelial progenitor cells through the dual activation mechanism of the phosphatidylinositol 3-kinase/Akt pathway, leading to vasculogenesis. Metabolically, PPAR-δ is a key regulator with the potential to therapeutically target multiple aspects of the metabolic syndrome. PPAR-δ activation was known to elevate high-density lipoprotein cholesterol robustly and lower low-density lipoprotein cholesterol and triglycerides. It was also reported to be involved in adipose tissue metabolism and to prevent obesity. In liver, PPAR-δ activation was shown to suppress hepatic glucose output, contributing to improved glucose homeostasis. Metabolic syndrome has been strongly linked with cardiovascular disease. Considering the beneficial effects of PPAR-δ agonist in metabolic syndrome, we can expect that its therapeutic potential may be enormous in cardiovascular disease on the basis of our new findings that PPAR-δ activation modulates endothelial progenitor cell biology in a positive direction and augments vasculogenesis.