Sirtuin 1 Mediates the Actions of Peroxisome Proliferator-Activated Receptor δ on the Oxidized Low-Density Lipoprotein–Triggered Migration and Proliferation of Vascular Smooth Muscle Cells

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ABSTRACT

Peroxisome proliferator-activated receptor δ (PPARδ) has been implicated in vascular pathophysiology. However, its functions in atherogenic changes of the vascular wall have not been fully elucidated. PPARδ activated by GW501516 [2-[2-methyl-4-[4-methyl-2-[4-(trifluoromethyl)phenyl]-1,3-thiazol-5-yl]methylsulfanyl]-phenoxy]acetic acid] significantly inhibited the migration and proliferation of vascular smooth muscle cells (VSMCs) triggered by oxidized low-density lipoprotein (oxLDL). These GW501516-mediated effects were significantly reversed by PPARδ-targeting small-interfering RNA (siRNA), indicating that PPARδ is involved in the action of GW501516. The antiproliferative effect of GW501516 was directly linked to cell cycle arrest at the G0/G1 to S phase transition, which was followed by the down-regulation of cyclin-dependent kinase 4 along with increased levels of p21 and p53. In VSMCs treated with GW501516, the expression of sirtuin 1 (SIRT1) mRNA and protein was time-dependently increased. This GW501516-mediated up-regulation of SIRT1 expression was also demonstrated even in the presence of oxLDL. In addition, GW501516-dependent inhibition of oxLDL-triggered migration and proliferation of VSMCs was almost completely abolished in the presence of SIRT1-targeting siRNA. These effects of GW501516 on oxLDL-triggered phenotypic changes of VSMCs were also demonstrated via activation or inhibition of SIRT1 activity by resveratrol or sirtinol, respectively. Finally, gain or loss of SIRT1 function imitated the action of PPARδ on oxLDL-triggered migration and proliferation of VSMCs. Taken together, these observations indicate that PPARδ-dependent up-regulation of SIRT1 contributes to the antiatherogenic activities of PPARδ by suppressing the migration and proliferation of VSMCs linked to vascular diseases such as restenosis and atherosclerosis.

INTRODUCTION

Oxidized low-density lipoprotein (oxLDL) is a proatherogenic risk factor that promotes the migration and growth of vascular smooth muscle cells (VSMCs) and macrophages in atherosclerotic lesions (Chatterjee and Ghosh, 1996; Sakai et al., 1996; Mine et al., 2002; Liu et al., 2014). VSMC proliferation and migration triggered by oxLDL were recently demonstrated as an important phenotypic change in multiple human vascular disorders such as atherosclerosis, and thus facilitating the formation and vulnerability of atherosclerotic plaque (Witztum and Steinberg, 2001). VSMCs play major roles in the maintenance of plaque stability as an important structural component of atherosclerotic plaque cap (Clarke and Bennett, 2006). Several studies have demonstrated that intimal migration and excess growth of VSMCs is a critical risk factor leading to restenosis and atherosclerosis (Stintzing et al., 2009; Brown et al., 2010). In fact, these phenotypic changes of VSMCs are regulated by interactions and networking between local cells and humoral factors within the arterial wall (Jiang et al., 2013). Thus, fine-tuned modulation of the migration and proliferation of VSMCs may have therapeutic potential in atherosclerotic complications.

Peroxisome proliferator-activated receptor δ (PPARδ) participates in multiple physiologic processes, including cellular energy homeostasis, cell growth and differentiation, and inflammatory responses as a nuclear transcription factor activated by specific ligands (Giordano Attianese and
Desvergne, 2015). This receptor heterodimerizes with retinoid X receptor in the consensus sequences named PPAR response elements (PPRE) to modulate the gene expression of target genes (Tugwood et al., 1992). It was recently observed that specific ligand-mediated activation of PPARδ in vascular cells exerts antiatherosclerotic effects through antiinflammatory mechanisms (Lee et al., 2003; Takata et al., 2008; Jandeleit-Dahm et al., 2009). Activation of PPARδ also suppresses proliferation and inflammation of VSMCs by up-regulating transforming growth factor-β1 (Kim et al., 2008). We have also demonstrated that ligand-activated PPARδ may stabilize atherosclerotic plaques by up-regulating extracellular matrix proteins in VSMCs (Kim et al., 2009). Furthermore, activation of PPARδ counteracts oxLDL-triggered apoptosis of VSMCs through transforming growth factor-β and focal adhesion kinase signaling (Hwang et al., 2015). Therefore, it is especially important to examine whether PPARδ modulates the phenotype of VSMCs induced by oxLDL.

Sirtuin 1 (SIRT1) functions as a protein deacetylase to remove the acetyl groups of diverse proteins in a NAD+-dependent manner (Michan and Sinclair, 2007). SIRT1 participates in multiple cellular processes such as modulation of the cell cycle, metabolism, and aging through interaction with diverse substrates including forkhead transcription factor, histones, MyoD, PPARγ coactivator-1α, and p53 (Vaziri et al., 2001; Langley et al., 2002; Brunet et al., 2004). Inhibitory effects of SIRT1 on the migration and growth of VSMCs were reported (Miyazaki et al., 2008; Li et al., 2011; Gorenne et al., 2013). Recently, a study reported that ligand-activated PPARδ enhances transcription of SIRT1 through Sp1 but not PPRE in HuH7 cells (Okazaki et al., 2010). Therefore, we hypothesized that the adverse effects of PPARδ on VSMC migration and growth may be in part stemmed by the up-regulation of SIRT1. Our findings indicate that PPARδ-induced up-regulation of SIRT1 mediates the ability of this transcription factor to inhibit the phenotypic changes of VSMCs induced by oxLDL.

Materials and Methods

Materials. GW501516 (GW501516 (2-[2-methyl-4-[4-(methyl-2-[4-(trifluoromethyl)phenyl]-1,3-thiazol-5-y)]methylsulfanyl)phenoxyl)acetic acid) and low-density lipoprotein were purchased from Enzo Life Science (Farmimgdale, NY) and Calbiochem (La Jolla, CA), respectively. Anti-β-actin antibody, 4,6-diamidino-2-phenylindole, mithramycin A, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and sirtuin were purchased from Sigma-Aldrich (St. Louis, MO). Resveratrol and GSK0660 (methyl 3-[[4-anilino-2-methoxyphenyl]sulfamoyl]thiopene-2-carboxylate) were obtained from Calbiochem and Tocris Bioscience (Bristol, United Kingdom), respectively. Polyclonal antibodies specific for horseradish peroxidase-conjugated IgG, c-Myc, PPARδ, and SIRT1 as well as monoclonal antibodies specific for cyclin D1, cyclin-dependent kinase (CDK) 2, p21, and p53 were from Santa Cruz Biotechnology (Dallas, TX). A monoclonal anti-CDK4 antibody was purchased from Cell Signaling (Beverly, MA).

Cell Culture. Aortic VSMCs were isolated and incubated in Dulbecco's modified Eagle's medium (DMEM) containing 1% antibiotic and 20% fetal bovine serum (FBS) at 37°C under a condition of 5% CO2 and 95% air, as essentially described by Hwang et al. (2015). Briefly, the aortic explants dissected from rats were longitudinally cut, and the endothelial cells were removed. The isolated membrane was chopped into small pieces and incubated for a few days in DMEM supplemented with 20% FBS. When VSMCs had fully migrated into the medium, the cells were maintained in the same culture medium.

Preparation of oxLDL. We prepared oxLDL as essentially described by Hwang et al. (2015). Briefly, native low-density lipoprotein (nLDL) was reacted with CuSO4 (final 5 μmol) at 37°C for 6 hours, and then 1 mM EDTA was added to stop the reaction. After thorough dialysis in phosphate-buffered saline, the extent of low-density lipoprotein oxidation was determined by measuring thiobarbituric reactive substances (Siow et al., 1999).

Small-Interfering RNA Study. Gene-specific small-interfering RNA (siRNA)-mediated gene silencing was performed as essentially described by Hwang et al. (2015). Briefly, VSMCs were transfected with nonspecific sequence-targeting siRNA (Ambion, Austin, TX), SIRT1-targeting siRNA (Bioneer, Daejeon, South Korea), and PPARδ-targeting siRNA (Ambion) using Welfect-Q (WelGENE, Daegeu, South Korea) in serum-free medium. The effects of siRNA were analyzed after incubation for 24 to 48 hours in fresh medium.

Assay of Cell Proliferation. VSMCs were synchronized to quiescence by incubating in DMEM containing 0.1% FBS for 24 hours. The cells were then treated with the indicated reagent for the indicated time in fresh DMEM containing 5% FBS, and exposed to nLDL or oxLDL. After incubation for 72 hours, the cells were maintained for an additional 4 hours in the culture medium containing MTI solution (final 0.1 mg/ml). After removal of the culture medium, the resultant formazan crystals were dissolved in acidified isopropanol, and the optical density was measured using a microplate reader at 570 nm as described elsewhere (Ham et al., 2013).

Cell Cycle Determination. VSMCs were synchronized to quiescence via incubation in 0.1% FBS-containing DMEM. After an overnight incubation, VSMCs were pretreated with GW501516 for 24 hours in fresh DMEM. Finally, the cells were incubated in DMEM containing oxLDL for an additional 72 hours, and then centrifuged at 1000 rpm for 5 minutes to collect the cells. The cells were then fixed by incubating with 70% (v/v) ethanol at −70°C for 1 hour. After washing with phosphate-buffered saline, a staining solution [10 mM Tris (pH 7.0), 0.1% NP-40, 1 mM NaCl, 0.7 μg/ml ribonuclease A, and 5 μg/ml propidium iodide] was added to the cells. The cellular DNA content was determined after incubation for 30 minutes in the dark, and cell cycle distributions were estimated with a FACS Calibur system (BD Biosciences, Franklin Lakes, NJ) and CellQuest Pro software (BD Biosciences).

Measurement of Cell Migration. A wound healing assay was adopted to measure VSMC migration as described elsewhere (Ham et al., 2014). To pause proliferation, VSMCs grown to confluency were adopted to measure VSMC migration as described elsewhere (Ham et al., 2013).

Real-Time Polymerase Chain Reaction. Expression of SIRT1 mRNA was analyzed by real-time polymerase chain reaction (PCR) as described elsewhere (Kim et al., 2013). Briefly, total RNA was isolated and reverse-transcribed into cDNA using TRIzol reagent (Invitrogen, Carlsbad, CA) and TOPOscript RT DryMIX kit (Enzynomics, Seoul, South Korea), respectively. An aliquot of cDNA was amplified in a PCR mix (Solgent, Daejeon, South Korea), followed by 40 cycles of PCR amplification (10 seconds at 95°C, 10 seconds at 56°C, and 10 seconds at 72°C). The primer sequences used were SIRT1F, 5’-AGA ACC ACC AAA GCC GAA A-3’ and 5’-TCC CAC AGG AGA CAG AAA CC-3’; and GAPDH, 5’-CAT GGC CCT CCG TGT TCC TA-3’ and 5’-CCT GCT TCA CCA CCT TCT TGA T-3’. The cDNA levels of the target gene were determined as the fold ratio relative to GAPDH by the ΔΔCT method (Hwang et al., 2014).
Western Blot Analysis. The levels of protein were analyzed by immunoblotting as described elsewhere (Hwang et al., 2015). In brief, whole cell lysates containing 50 μg of protein per lane were fractionated on a 10% SDS-polyacrylamide gel and blotted onto a polyvinylidene difluoride membrane (Hybond-P, 0.45 μm pore; Amersham Biosciences, Buckinghamshire, United Kingdom). After blocking with 5% skim milk, the membranes were probed with specific antibodies (anti-PPARδ diluted to 1:500; anti-SMAD1 diluted to 1:1000; anti-p53 diluted to 1:500; anti-p21 diluted to 1:500; anti-CDK4 diluted to 1:1000; anti-CDK2 diluted to 1:1000; anti-cyclin D diluted to 1:500; anti-β-actin diluted to 1:3000) overnight at 4°C. After incubation with a peroxidase-conjugated secondary antibody, the immunoreactive band was visualized by conjunction with West-ZOL Plus (INTRON Bio-technology, Seongnam, South Korea).

Vector Constructs. Recombinant adenoviral vectors were constructed for lacZ and Myc-SIRT1 (GenBank: AF083106) expression. All cDNAs were ligated into the pAd/CMV/V5-DEST vector (Invitrogen) using LR Clonase II and Gateway system (Invitrogen). Recombinant adenoviral vector was linearized with PacI and transferred into 293A cells, and then cultured for 2 weeks in complete DMEM. The pAd/CMV/GW5-lacZ vector (Invitrogen) as a control was also introduced to produce a lacZ-bearing adenovirus. The KIST virus facility (Seoul, Korea) was used to produce adenoviruses. The mammalian expression vector pcDNA3.1-Flag-Sp1 was presented by Prof. Jae-Hwan Kim (CHA University, Pungyo, South Korea).

Reporter Gene Assay. The PPRE3-k-luc luciferase reporter vector was a gift from Prof. Frank J. Gonzalez (National Cancer Institute, National Institutes of Health, Bethesda, MD). VSMCs transfected with 0.5 μg pcDNA3.1–PPARδ, 0.5 μg pTK-luc, 0.5 μg pSV-β-gal, and/or 0.5 μg pcDNA3.1–Myc-SIRT1 for 48 hours were incubated with or without GW501516 and/or resveratrol for the indicated times. The cells were then lysed, and the reporter gene activity was measured as described elsewhere (Hwang et al., 2015).

Statistical Analysis. One-way analysis of variance followed by the Tukey-Kramer test was used to determine statistical significance.

Results

Activation of PPARδ Inhibits oxLDL-Induced Proliferation of VSMCs. Because oxLDL increases the proliferation of cultured VSMCs (Chatterjee and Ghosh, 1996), we assessed whether activation of PPARδ by GW501516 affects the VSMC growth stimulated by oxLDL. A concentration-dependent increase in proliferation was demonstrated in VSMCs treated with oxLDL relative to nLDL-treated cells, as determined by the MTT assay (Fig. 1A). By contrast, the oxLDL-stimulated VSMC proliferation was dose-dependently inhibited in cells treated with GW501516, a specific ligand for PPARδ (Fig. 1B).

To investigate the involvement of PPARδ in the inhibition of VSMC proliferation triggered by oxLDL, VSMCs were treated with PPARδ-targeting siRNA or GSK0660, a PPARδ-specific antagonist (Shearer et al., 2008). The expression of PPARδ protein was reduced in VSMCs transfected with PPARδ-targeting siRNA, whereas nonspecific control siRNA did not (Supplemental Fig. 1). The addition of PPARδ-targeting siRNA or GSK0660 significantly reversed the inhibitory effect of GW501516 on VSMC proliferation triggered by oxLDL (Fig. 1, C and D).

To further characterize the antiproliferative action of PPARδ in oxLDL-treated VSMCs, the cell cycle distribution was analyzed in VSMCs treated with or without oxLDL in the presence or absence of GW501516. The cells were synchronized by incubating in medium containing 0.1% FBS for 24 hours, and then exposed to vehicle (dimethylsulfoxide) or GW501516, followed by stimulation with oxLDL. A significant decrease in the G2/G1 population was observed in VSMCs exposed to oxLDL for 72 hours (Fig. 2, A and B). By contrast, the population of cells proceeding the S phase was significantly increased. GW501516, however, significantly reversed the oxLDL-induced reduction in cell populations in the G2/G1 phase. Especially, the increased transition of oxLDL-stimulated cells into the S phase was also significantly suppressed by G501516. These findings suggest that activation of PPARδ by GW501516 causes the cell cycle arrest of VSMC treated with oxLDL.

Because a dynamic network of cell cycle–related molecules such as CDKs, cyclins, and CDK inhibitors is associated with regulation of the cell cycle (Pavletich, 1999), we examined the expression levels of key regulatory proteins in VSMCs treated with oxLDL in the presence or absence of GW501516. We found that oxLDL markedly suppressed the levels of p21 and p53 (Fig. 2, C and D), key regulators of the G1 to S phase transition checkpoint (Toyoshima and Hunter, 1994; Pavletich, 1999). This oxLDL-dependent decrease in both protein levels was significantly reversed in the presence of GW501516. In addition, up-regulation of CDK4, but not CDK2, induced by oxLDL was also significantly down-regulated by GW501516. These observations provide evidence that GW501516-activated PPARδ arrests oxLDL-induced cell cycle progression through expression modulation of cell cycle–related proteins.

Activation of PPARδ Inhibits oxLDL-Stimulated Migration of VSMCs. Next, we investigated the role of GW501516 on oxLDL-triggered migration of VSMCs by an in vitro wound healing assay. With the proliferation index as a reference basis, described previously elsewhere (Kim et al., 2010), we found that oxLDL significantly enhanced the migration of VSMCs whereas this effect of oxLDL on cell migration was significantly suppressed by GW501516.
treatment (Fig. 3, A and B). GW501516-mediated reduction in VSMC migration was not observed in cells transfected with PPARδ-targeting siRNA (Fig. 3, C and D). These observations suggest that the action of GW501516 is dependent on PPARδ in the suppression of VSMC migration triggered by oxLDL.

**Activation of PPARδ Induces SIRT1 Expression in VSMCs.** Because SIRT1 is implicated in neointima formation through modulation of VSMC migration and proliferation (Li et al., 2011), the expression level of SIRT1 was determined in VSMCs treated with GW501516. The levels of both SIRT1 mRNA and protein in VSMCs were significantly increased by GW501516 treatment in a time-dependent manner (Fig. 4, A and B).

To confirm whether this effect of GW501516 on the up-regulation of SIRT1 is PPARδ dependent, the effects of PPARδ-targeting siRNA and GSK0660 were examined. PPARδ-targeting siRNA, but not nonspecific control siRNA, markedly inhibited GW501516-induced up-regulation of SIRT1.

**Fig. 2.** GW501516-activated PPARδ regulates cell cycle progression of VSMCs triggered by oxLDL. (A, B) Cells synchronized to quiescence by incubating in DMEM containing 0.1% FBS were pretreated with GW501516 for 24 hours. After incubation for 72 hours in DMEM containing 5% FBS and oxLDL, the DNA content in cells stained with propidium iodide was analyzed by flow cytometry. Representative histograms (A) and the cell number of each cell cycle (B) are plotted as mean ± S.E. (n = 3). (C, D) The expression of cell cycle-regulatory proteins was detected by Western blot analysis. Representative blots (C) and the fold changes in each protein to β-actin ratio relative to untreated control (D) are shown as mean ± S.E. (n = 4). *P < 0.05, **P < 0.01 versus 5% serum-treated cells; #P < 0.01, ##P < 0.05 versus oxLDL-treated cells.

**Fig. 3.** GW501516-activated PPARδ attenuates oxLDL-triggered migration of VSMCs. (A, B) Mitomycin C (8 μg/ml)-treated cell monolayers were incubated for 2 hours, and then washed with phosphate-buffered saline. After pretreatment with or without GW501516 for 24 hours, the cells were wounded by scraping with a razor blade, and then stimulated with either nLDL or oxLDL in fresh medium containing 5% FBS. After incubation for 72 hours, a wound healing assay was performed to count the cells that migrated across the wound. (C, D) Cells transfected with PPARδ-targeting siRNA were treated as described earlier, and a wound healing assay was performed as described herein. Representative photographs (A, C) and migrated cell numbers (B, D) are shown as mean ± S.E. (n = 6). Bars: 100 μm. *P < 0.05, **P < 0.01 versus untreated control; †P < 0.05 versus oxLDL plus GW501516-treated cells.
SIRT1 in VSMCs (Fig. 4C). In addition, the effect of GW501516 on SIRT1 expression was also suppressed by GSK0660 pretreatment (Fig. 4D), indicating the involvement of PPARβ in the up-regulation of SIRT1.

Because Sp1 is implicated in PPARβ-mediated SIRT1 expression (Okazaki et al., 2010), the effect of mithramycin A, a specific Sp1 inhibitor, was examined in PPARβ-mediated SIRT1 expression. When VSMCs were incubated in the presence of mithramycin A, GW501516-induced up-regulation of SIRT1 was markedly inhibited (Fig. 4E). This effect of mithramycin A was also observed in GW501516-mediated suppression of VSMC proliferation triggered by oxLDL (Supplemental Fig. 2A). In addition, ectopic expression of Sp1 potentiated the suppressive effect of GW501516 on the VSMC proliferation triggered by oxLDL (Supplemental Fig. 2B). These findings indicate that GW501516-regulated SIRT1 is an important factor in the modulation of VSMC proliferation stimulated by oxLDL.

On the other hand, SIRT1 has been reported to inhibit the transcriptional activity of PPARγ (Picard et al., 2004), another member of PPAR nuclear family, so we assessed the effects of SIRT1 overexpression and activation on the transcriptional activity of PPARβ. Although GW501516 significantly increased the transcriptional activity of PPARβ, the ectopic expression of SIRT1 or addition of resveratrol did not significantly affect the GW501516-activated increase in the transcriptional activity of PPARβ, which suggests that SIRT1 did not directly influence the transcriptional activity of PPARβ in VSMCs (Supplemental Fig. 3).

**Down-Regulation of SIRT1 Abrogates the Inhibitory Effect of PPARβ on VSMC Migration and Proliferation Triggered by oxLDL.** Because the expression of SIRT1 was up-regulated in VSMCs by PPARβ activation, we examined the role of SIRT1 in PPARβ-associated modulation of cell migration and proliferation in VSMCs stimulated with oxLDL. The expression of SIRT1 was time-dependently decreased in the presence of oxLDL, but this oxLDL-triggered reduction of SIRT1 was dose-dependently reversed by GW501516 (Fig. 5, A and B).

To further examine whether this GW501516-mediated recovery of SIRT1 expression directly affects oxLDL-stimulated migration and proliferation of VSMCs, we examined the effect of SIRT1-targeting siRNA in VSMCs (Supplemental Fig. 4). Although GW501516 significantly inhibited oxLDL-stimulated migration and proliferation of VSMCs, siRNA-mediated knockdown of SIRT1 in VSMCs almost completely abolished the actions of GW501516 on the migration and proliferation of VSMCs triggered by oxLDL. (A) Cells were treated with oxLDL for the time period indicated. (B) GW501516-pretreated cells were incubated for 24 hours and, then were exposed to oxLDL for 72 hours. Immunoblot analysis was performed to detect the expression of SIRT1 protein using specific antibodies for SIRT1 and β-actin. (C) SIRT1 siRNA-transfected cells were incubated for 24 hours, and then were pretreated with or without GW501516. After incubation for 24 hours, the transfectants were exposed to oxLDL for 72 hours. An MTT assay was performed to determine cell proliferation. (D) SIRT1 siRNA-transfected cell monolayers were incubated for 24 hours, and then were pretreated consecutively with or without mitomycin C (8 μg/ml) and/or GW501516 for 2 and 24 hours, respectively, before exposure to oxLDL for 72 hours. A wound healing assay was performed to count the cells that migrated across the wound, and is expressed as mean ± S.E. (n = 6). *P < 0.05, **P < 0.01 versus untreated cells; #P < 0.05 versus oxLDL-treated cells; †P < 0.05 versus oxLDL plus GW501516-treated cells.
The effect of GW501516 on oxLDL-triggered migration and proliferation (Fig. 5, C and D). These observations suggest that PPARδ inhibits oxLDL-triggered cell migration and proliferation through modulation of SIRT1 expression.

**Ectopic Overexpression of SIRT1 Mimics the Suppressive Action of PPARδ in VSMC Migration and Proliferation Triggered by oxLDL.** To further characterize the critical role of SIRT1 in oxLDL-triggered cell migration and proliferation, the effects of SIRT1 overexpression were examined in VSMCs. The ectopic expression of SIRT1 increased the level of SIRT1 in VSMCs (Supplemental Fig. 5). In line with the levels of SIRT1 expression, the oxLDL-triggered migration and proliferation of VSMCs were significantly attenuated, indicating that SIRT1 is an important protein in PPARδ-mediated inhibition of VSMC migration and proliferation triggered by oxLDL (Fig. 6).

**SIRT1 Is an Important Factor in PPARδ-Mediated Suppression of VSMC Migration and Proliferation Triggered by oxLDL.** To confirm the functional significance of PPARδ-mediated induction of SIRT1, we investigated the impact of SIRT1 activity on PPARδ-mediated inhibition of VSMC migration and proliferation in cells triggered by oxLDL. Blockade of SIRT1 activity by sirtinol, a specific SIRT1 inhibitor, reversed the inhibitory actions of PPARδ on the VSMC migration and proliferation triggered by oxLDL (Fig. 7; Supplemental Fig. 6). By contrast, resveratrol, a SIRT1 activator, significantly inhibited both oxLDL-stimulated cell migration and proliferation to a similar degree as GW501516 did in VSMCs (Fig. 7). This inhibitory effect of GW501516 was further enhanced in the presence of resveratrol, suggesting that the actions of PPARδ on the VSMC migration and proliferation are mediated through expression regulation of SIRT1 (Fig. 7; Supplemental Fig. 6).

**Discussion**

Transcriptional activation of specific target genes by PPARδ elicits a broad range of cellular responses to maintain vascular homeostasis (Lee et al., 2003; Kim et al., 2008, 2009). Although diverse target genes participate in the vascular actions of PPARδ, the effector molecules associated with the vascular actions of PPARδ have not been fully elucidated. Here, we showed that a specific ligand GW501516-activated PPARδ inhibits oxLDL-stimulated migration and proliferation of VSMCs, which trigger formation of a fibrous capsule in the pathogenesis of atherosclerosis (Dzau et al., 2002; Gorenne et al., 2013). This PPARδ-mediated inhibition is dependent on SIRT1, a deacetylase that has a multitude of roles in the modulation of cardiovascular diseases (Ota et al., 2010). In VSMCs treated with GW501516, the mRNA and protein of SIRT1 were up-regulated. Chemical- or siRNA-mediated modulation of SIRT1 activity or expression abrogated the inhibitory actions of PPARδ on the migration and proliferation of VSMCs triggered by oxLDL. Furthermore, adenovirus-mediated ectopic expression of SIRT1 potentiated these effects of GW501516 on VSMCs.

The antiatherogenic properties of PPARδ are, in part, associated with its activity to induce the intracellular multifunctional gene SIRT1 in VSMCs. Our present observations are consistent with a previous finding demonstrating that activation of PPARδ suppresses premature senescence by up-regulating SIRT1 in human endothelial cells exposed to angiotensin II (Kim et al., 2012). In addition, SIRT1 transgenic mice exhibited protection against atherosclerosis via inhibition of VSMC migration and proliferation, which underlie neointima formation (Li et al., 2011). By contrast, another study showed that SIRT1 promoted proliferation by...
preventing senescence of primary endothelial cells cultured from porcine aorta (Zu et al., 2010).

Although the action of SIRT1 in cell proliferation is debatable, this study demonstrates that activation of PPARδ inhibits oxLDL-stimulated VSMC migration and proliferation. In human atherosclerotic plaques, the reduced expression of SIRT1 has also been detected (Gorenne et al., 2013); therefore, it may be possible to control the stability of plaques through SIRT1 up-regulation in a process mediated by PPARδ activation. Our current observations provide new clues in the role of PPARδ as a potential novel therapeutic target in cardiovascular diseases associated with VSMC migration and proliferation such as atherosclerosis and restenosis.

Reduced expression of SIRT1 was demonstrated in VSMCs isolated from plaques and human atherosclerotic plaques (Gorenne et al., 2013). In accordance with results in atherosclerotic plaques, our study has demonstrated that the expression of SIRT1 in VSMCs was markedly suppressed by exposure to oxLDL, which is implicated in various aspects of cardiovascular diseases, in particular atherosclerosis (Trpkovic et al., 2015). This oxLDL-induced suppression of SIRT1 expression was dose-dependently reversed by the activation of PPARδ in VSMCs. In fact, the direct modulatory properties of the migration and proliferation of VSMCs induced by oxLDL were demonstrated by siRNA- or adenovirus-mediated modulation of SIRT1 expression as well as chemical-mediated modulation of SIRT1 activity. Accordingly, oxLDL-induced down-regulation of SIRT1 seems to be involved in the pathogenesis of vascular disorders triggered by oxLDL.

Our finding show that activation of PPARδ significantly enhances SIRT1 expression regardless of oxLDL in VSMCs. Although the role of PPARδ in SIRT1 expression was not directly evaluated, the Sp1 inhibitor mithramycin A clearly inhibited the GW501516-induced expression of SIRT1. This finding was in line with reported results showing that ligand-activated PPARδ transcriptionally activates the SIRT1 gene through Sp1 in VSMCs and hepatocyte-derived HepH7 (Okazaki et al., 2010; Kim et al., 2012). Our present results indicate that Sp1 is a critical factor in the PPARδ-mediated induction of SIRT1 expression instead of PPRE.

In addition, Sp1 also inhibits VSMC proliferation by transcriptionally increasing mitofusin-2 or growth inhibitory protein p27 (Andrés et al., 2001; Sorianello et al., 2012). In line with the previous reports, modulation of Sp1 by inhibitor or ectopic overexpression significantly affected to the actions of GW501516 on the VSMC proliferation triggered by oxLDL. SIRT1 also inhibits vascular injury–induced cell proliferation in carotid artery-ligated mice (Li et al., 2011), which stresses the importance of SIRT1 in restenosis. In fact, up-regulation of SIRT1 by PPARδ inhibits platelet-derived growth factor–or interleukin-β–induced migration and proliferation of VSMCs in a cell cycle–dependent manner (Lim et al., 2009; Kim et al., 2010). Furthermore, activation or inhibition of SIRT1 activity by resveratrol or sirtinol, respectively, modulated VSMC migration and proliferation triggered by oxLDL in our present experimental conditions. These observations indicate that PPARδ inhibits oxLDL-triggered migration and proliferation of VSMCs through a mechanism linked to SIRT1.

PPARδ-dependent up-regulation of SIRT1 is a key event in the inhibition of VSMC migration and proliferation triggered by oxLDL. SIRT1, a NAD+–dependent histone deacetylase, has a variety of cellular functions in cell cycle regulation, organism longevity, and energy metabolism (Finkel et al., 2009). Our study showed that GW501516-activated PPARδ suppressed oxLDL-triggered migration and proliferation of VSMCs. This effect of PPARδ on VSMC migration and proliferation was linked to the levels of SIRT1, indicating that SIRT1 is a critical factor in the modulation of atherosclerotic pathogenesis (Li et al., 2011; Gorenne et al., 2013). Thus, SIRT1 has emerged as an important modulator that promotes vascular functions (Li et al., 2011; Gorenne et al., 2013).

Such findings provide a rationale for SIRT1 as a drug target in vascular disorders, including restenosis and atherosclerosis. In fact, many other studies have provided evidence that the level of SIRT1 expression is intimately linked to atherosclerotic vascular disorders (Li et al., 2011; Kim et al., 2012; Gorenne et al., 2013). Because down-regulated level of SIRT1 has been demonstrated in VSMCs of the atherosclerotic plaque region (Gorenne et al., 2013), it may be possible to inhibit the proatherogenic effect of decreased SIRT1 in the vascular wall by inducing SIRT1 expression through activation of PPARδ.

In conclusion, SIRT1 was identified as a key molecular target of PPARδ-mediated antimigration and antiproliferation actions in VSMCs stimulated with oxLDL. These observations indicate that GW501516-activated PPARδ inhibits the migration and proliferation of VSMCs triggered by oxLDL by modulating SIRT1 expression, suggesting that reinforcement of SIRT1 expression by PPARδ could be a novel therapeutic strategy for vascular disorders such as restenosis and atherosclerosis.

Authorship Contributions

Participated in research design: Hwang, Seo.

Conducted experiments: Hwang, Ham, Yoo, W.J. Lee.


Wrote or contributed to the writing of the manuscript: Hwang, Paek, Seo.

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