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# Anti-Proliferative Activity of OD78 Is Mediated through Cell Cycle Progression by Upregulation $p27^{kip1}$ in Rat Aortic Vascular Smooth Muscle Cells

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## Abstract

Atherosclerosis and post-angiography restenosis are associated with intimal thickening and concomitant vascular smooth muscle cell (VSMC) proliferation. Obovatol, a major biphenolic component isolated from the *Magnolia obovata* leaf, is known to have anti-inflammatory and anti-tumor activities. The goal of the present study was to enhance the inhibitory effects of obovatol to improve its potential as a preventive or therapeutic agent in atherosclerosis and restenosis. Platelet-derived growth factor (PDGF)-BB-induced proliferation of rat aortic smooth muscle cells (RASMCs) was examined in the presence or absence of a newly synthesized obovatol derivative, OD78. The observed anti-proliferative effect of OD78 was further investigated by cell counting and [$^3$H]-thymidine incorporation assays. Treatment with 1-4 μM OD78 dose-dependently inhibited the proliferation and DNA synthesis of 25 ng/ml PDGF-BB-stimulated RASMCs. Accordingly, OD78 blocked PDGF-BB-induced progression from the $G_0/G_1$ to S phase of the cell cycle in synchronized cells. OD78 decreased the expression levels of CDK4, cyclin E, and cyclin D1 proteins, as well as the phosphorylation of retinoblastoma protein and proliferating cell nuclear antigen; however, it did not change the CDK2 expression level. In addition, OD78 inhibited downregulation of the cyclin-dependent kinase inhibitor (CKI) $p27^{kip1}$. However, OD78 did not affect the CKI $p21^{cip1}$ or phosphorylation of early PDGF signaling pathway. These results suggest that OD78 may inhibit PDGF-BB-induced RASMC proliferation by perturbing cell cycle progression, potentially through $p27^{kip1}$ pathway activation. Consequently, OD78 may be developed as a potential anti-proliferative agent for the treatment of atherosclerosis and angioplasty restenosis.

**Key Words:** Smooth muscle cell, PDGF-BB, $p27^{kip1}$, Cell cycle, Proliferation

## INTRODUCTION

The abnormal migration and proliferation of vascular smooth muscle cells (VSMCs) in arterial walls are important pathogenetic factors of vascular disorders such as atherosclerosis and post-angioplasty restenosis (Ross, 1993). Vascular lesions are formed during several pathological processes involving growth factor release. Platelet-derived growth factor (PDGF)-BB is a potent growth factor produced by platelets, VSMCs, and endothelial cells in the injured vascular wall (Majesky *et al.*, 1990; Miyauchi *et al.*, 1998). PDGF initiates the activation of intracellular signal transduction pathways that contribute to VSMC proliferation, migration, and collagen synthesis (Ammon and Wahl, 1991). PDGF-BB propagates mitogenic signals through the autophosphorylation of its respective PDGF beta-receptor (Rβ) on tyrosine residues, thus triggering downstream signal transduction and cell cycle progression (Blenis, 1993; Heldin *et al.*, 1998; Ahn *et al.*, 1999).

Cell cycle phases are coordinated by the expression and activation of regulatory proteins, including complexes of cyclins and cyclin-dependent kinases (CDK) (Braun-Dullaeus *et al.*, 1998). The kinase activity of these CDK-cyclin complexes is further negatively regulated by two classes of cyclin-dependent kinase inhibitors (CKIs) (Sherr and Roberts, 1999). INK4 family members ($p16^{INK4a}$ and $p15^{INK4b}$) inhibit only CDK4 and CDK6 (Ortega *et al.*, 2002), while cip family members ($p21^{cip1}$

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and p27$^{kip1}$) inhibit all CDKs (Coqueret, 2003). CKI, a critical determinant for cell cycle progression, is an important regulatory target of mitogenic signals during arterial injury (Claesson-Welsh, 1994).

Obovatol has diverse anti-inflammatory, anti-tumor, muscle relaxation, anti-allergic, anti-bacterial, and neurite-sprouting activities (Ito *et al.*, 1982; Hwang *et al.*, 2002; Pyo *et al.*, 2002; Choi *et al.*, 2007; Lee *et al.*, 2008a; Choi *et al.*, 2009; Lee *et al.*, 2009). Synthesized obovatol derivatives can reportedly inhibit PDGF-BB-induced rat aortic smooth muscle cell (RASMC) proliferation (Yu *et al.*, 2009). The beneficial effects of obovatol may be due to its inhibitory potential against abnormal cell proliferation (Lim *et al.*, 2010). The purpose of this study was to increase the bioavailability of a newly developed obovatol derivative, OD78, while preserving its beneficial activities. We attempted to elucidate the anti-proliferative activity and machinery targets of OD78 in the PDGF-BB-induced signaling pathway. The results provide evidence that OD78 can inhibit proliferation and cell cycle progression via cell cycle-related proteins by regulating p27$^{kip1}$ in RASMCs.

## MATERIALS AND METHODS

### Materials and reagents

The obovatol derivative OD78 [3-(4-Bromo-phenoxy)-4,5-dihydroxy-benzoic acid methyl ester] was synthesized. Bovine serum albumin (BSA) and other chemicals were from Sigma Chemical Co. (St. Louis, MO, USA). Cell culture materials were purchased from Gibco-BRL (Gaithersburg, MD, USA). [$^3$H]-thymidine was purchased Amersham Pharmacia Biotech (Buckingamshire, UK). Extracellular signal-regulated kinase 1/2 (ERK1/2), phospho-ERK1/2, phospho-Akt, Akt, and phospho-retinoblastoma protein (phospho-Rb) antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). PDGF-BB, anti-phospho-PDGF-Rβ and anti-PDGF-Rβ were obtained from Upstate Biotechnology (Lake Placid, NY, USA). Cyclin D1, cyclin E, CDK2, CDK4, α-actin, proliferating cell nuclear antigen (PCNA), p21$^{cip1}$, and p27$^{kip1}$ were purchased from Santa Cruz Biotechnology Inc. (San Jose, CA, USA). All other chemicals were of analytical grade.

### Synthesis and characterization of OD78

Infrared or $^1$H NMR spectra were recorded on a Jasco FT-IR 300E or Bruker DPS300 spectrometer, respectively. Chemical shifts are expressed in parts per miliom (ppm, ð) downfield from tetramethylsilane. Reagents were purchased from Aldrich Chemical Co. (St. Louis, MO, USA). 3-(4-Bromo-phenoxy)-4,5-dihydroxy-benzoic acid methyl ester (OD78) was prepared by coupling 4-bromo-phenylboronic acid with a phenolic compound and Cu(OAc)$_2$, according to a method reported elsewhere (Lee *et al.*, 2008b). OD78 (Fig. 1): IR (thin film) 3,396, 1,698 cm $^{-1}$; $^1$H NMR (CD$_3$OD, 300 MHz) ð 7.44 (d, 2H, *J*=8.9Hz), 7.33 (s, IH), 7.11 (s, 1H), 6.85 (d, 2H, *J*=8.9Hz), 3.81 (s, 3H).

### Cell culture

RASMCs were isolated by enzymatic dispersion according to the modified method (Lim *et al.*, 2007). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 μg/ml streptomycin, 8 mM HEPES, and 2 mM L-glutamine

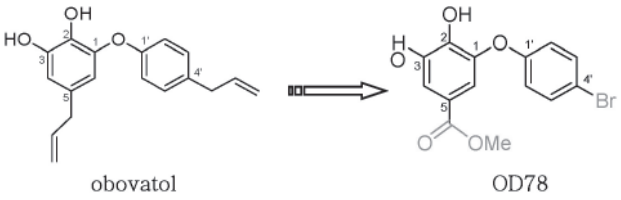


**Fig. 1.** Chemical structures of obovatol and its synthetic derivative, OD78. The structures of obovatol and OD78 were identified as 5-allyl-3-(4-allyl-phenoxy)-benzene-1,2-diol and 3-(4-bromo-phenoxy)-4,5-dihydroxy-benzoic acid-methyl ester, respectively.

at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$. Purity of the RASMC culture was confirmed by immunocytochemical localization of α-SM actin. RASMCs were used at the 4$^{th}$ to 8$^{th}$ passage.

### Cell viability assay

RASMCs were seeded into 96-well culture plates at 2×10$^4$ cells/ml and then cultured in DMEM containing 10% FBS at 37°C for 24 h. When cells reached 70% confluence, the medium was replaced with serum-free medium. After incubation for 24 h, the cells were exposed to 4 μM OD78 or 100 μg/mL digitonin. At various times, WST-1 reagent was added to and incubated with the cultures for 4 h. The absorbance was measured at 450 nm using a microplate reader (Packard Instrument Co., Downers Glove, IL, USA).

### Cell proliferation assay

The RASMCs were seeded into 12-well culture plates at 4×10$^4$ cells/mL and cultured in DMEM containing 10% FBS at 37°C for 24 h. When the cells reached 70% confluence, the medium was replaced with serum-free medium containing various concentrations of OD78. The cells were incubated for 24 h, stimulated with 25 ng/ml PDGF-BB, and incubated further for 24 h. The cells were trypsinized with trypsin-EDTA and counted using a hemocytometer under a light microscope.

### DNA synthesis assay

DNA synthesis was determined by [$^3$H]-thymidine incorporation, as previously described (Kim *et al.*, 2005). Briefly, RASMCs were seeded in 24-well plates. The medium was replaced with fresh serum-free medium and the cells were incubated for 24 h. The cells were incubated with various concentrations of OD78 for 24 h, stimulated with 25 ng/ml PDGF-BB for 20 h, and 2 μCi/ml of [$^3$H]-thymidine was added to the medium for the final 4 h. Reactions were terminated by aspirating the medium, and the cultures were sequentially washed on ice with PBS containing 10% trichloroacetic acid and ethanol/ether (1:1, v/v). Acid-insoluble [$^3$H]-thymidine was extracted into 500 μl of 0.5 M NaOH/well. The solution was mixed with 3 ml scintillation cocktail (Ultimagold, Packard Bioscience, Meriden, CT, USA) and quantified using a liquid scintillation counter (LS3801, Beckman, Düsseldorf, Germany).

### Cell cycle progression analysis

Cell cycle was determined as previously described (Kim *et al.*, 2005). Briefly, RASMCs in a 60-mm$^2$ cell culture dish were incubated for 24 h in serum-free DMEM medium (Gibco-BRL, Rockville) in the presence or absence of 1, 2, or 4 μM OD78. The RASMCs were stimulated with 25 ng/ml PDGF-BB for

24 h, trypsinized, and centrifuged at 1,500×g for 7 min. Centrifuged pellets were suspended in 1 ml of 1×PBS, washed twice, suspended in 70% ethanol, and fixed overnight at 4°C. The fixed cells were briefly vortexed and centrifuged at 15,000 ×g for 5 min. The ethanol was discarded, and the pellets were stained with 0.4 ml of propidium iodide (PI) solution (50 μg/ml PI in sample buffer containing 100 μg/ml of RNase A). Before flow cytometry, each sample was incubated at room temperature for 1 h. The PI-DNA complex in each cell nucleus was measured with a FACS Calibur (Becton & Dickinson Co.). The cell cycle rate within the $G_0/G_1$, S, or $G_2$/M phase was determined by analysis with Modfit LT software (Verity Software House, Inc.).

### Western blot assay

Immunoblotting was performed as previously described (Kim et al., 2005). Briefly, RASMCs were seeded in 6-well culture plates at $4\times10^4$ cells/mL and cultured in DMEM with 10% FBS at 37°C for 24 h. When cells reached 70% confluence, the cells were incubated in new serum-free medium for 24 h. The medium was changed to serum-free medium with various concentrations of OD78, and the cells were incubated for 24 h. RASMCs were stimulated with 25 ng/ml PDGF-BB for 1 min (for PDGF-Rβ), 5 min (for ERK1/2 and PLCγ1), or 15 min (for Akt).

To assay for cyclin D1, cyclin E, CDK2, CDK4, PCNA, $p21^{cip1}$, and $p27^{kip1}$ expression or pRb phosphorylation, RASMCs were stimulated with 25 ng/ml PDGF-BB for 24 h. After removing the medium, the cells were lysed with SDS lysis buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM dithiothreitol (DTT) and protease inhibitor Cocktail Tablet (Roche, Mannheim, Germany). The lysates were centrifuged at 12,000×g for 10 min and the supernatants were collected.

Proteins were separated in 7.5-15% SDS polyacrylamide gel (SDS-PAGE) using a Mini-Protein III System (Bio-Rad, CA, USA) and transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia Biotech) at 250 mA with a transfer buffer containing 25 mM Tris-HCl, 192 mM glycine, and 20% methanol (pH 8.3). The PVDF membrane was blocked with 5% BSA in TBS-T at room temperature for 4 h. The membranes were washed using TBS-T, incubated overnight with primary antibodies at 4°C, washed, and incubated with horseradish peroxidase-conjugated IgG secondary antibody (New England Biolabs, MA, USA) at 1:5000 dilution in BSA/TBS-T buffer at room temperature for 2 h.

After washing, the immunoreactive proteins were detected by chemiluminescent reaction (ECL plus kit, Amersham Pharmacia Biotech) and the membranes were exposure to Hyperfilm ECL (Amersham Pharmacia Biotech). The detected proteins were normalized by α-actin or the respective total proteins, respectively. The band intensities were quantified using a Scion-Image for Window Program (Scion Corporation, MA, USA).

### Statistical analysis

Experimental results are expressed as the mean ± S.E.M. One-way analysis of variance (ANOVA) followed by Dunnett's test was used for multiple comparisons. Statistically significant differences were defined at the $p<0.05$ or $p<0.01$ level.

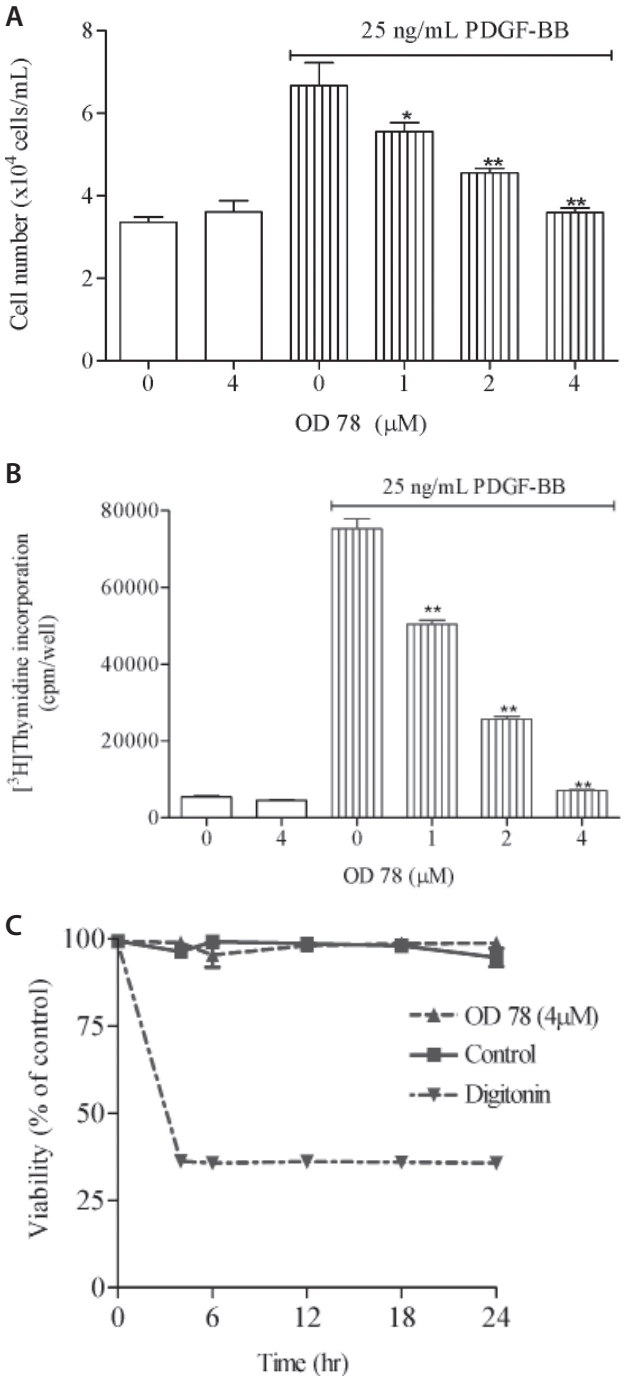


**Fig. 2.** Effect of OD78 on PDGF-BB-stimulated proliferation and DNA synthesis of RASMCs. (A) For the cell proliferation assay, RASMCs were precultured in serum-free medium in the presence or absence of 1, 2, or 4 μM OD78 for 24 h and then stimulated with 25 ng/ml PDGF-BB for 24 h. The cells were trypsinized and counted. (B) For the DNA synthesis assay, RASMCs were sequentially cultured sequentially in serum-starved medium in the presence or absence of OD78 (1, 2, or 4 μM) for 24 h, 25 ng/ml PDGF-BB for 24 h, and with 2 uCi/ml [$^3$H]-thymidine for 4 h. The labeling reaction was terminated, and radioactivity was quantified using a liquid scintillation counter. (C) RASMCs were exposed to 4 μM OD78 or 100 μg/ml digitonin. At the indicated times (6, 12, 18 and 24 h), cells were processed for their viability by the WST-1 assay. Data represent the mean ± S.E.M. from four independent sets of experiments. *$p<0.05$ and **$p<0.01$ vs. RASMCs stimulated with PDGF-BB alone.

## RESULTS

### Inhibitory effect of OD78 on PDGF-BB-induced RASMC proliferation

Direct cell counting was used to determine whether OD78 inhibited PDGF-BB-stimulated RASMC proliferation. Treatment of RASMCs with 25 ng/mL PDGF-BB for 24 h increased the number of cells from 3.5 ± 0.1×$10^4$ to 6.9 ± 0.3×$10^4$ cells/well. Incubation with both PDGF-BB and 1, 2, or 4 μM OD78 for 24 h dose-dependently decreased the number of cells to 5.6 ± 0.2, 4.6 ± 0.1, and 3.3 ± 0.2×$10^4$ cells/well, respectively, compared to treatment with PDGF-BB alone (Fig. 2A).

### Effect of OD78 on PDGF-BB-induced DNA synthesis

The effect of OD78 on DNA synthesis and cell proliferation was assayed using [$^3$H]-thymidine incorporation. When RASMCs were stimulated with 25 ng/ml PDGF-BB, the radioactivity of the synthesized DNA was increased (Fig. 2B). Treatment with 1, 2, or 4 μM OD78 significantly inhibited PDGF-BB-stimulated DNA synthesis by 34.6 ± 0.7, 66.6 ± 1.3, and 90.5 ± 0.6%, respectively. The DNA synthesis in RASMCs at 4 μM OD78 was reduced to the level in non-stimulated cells. Treatment of RASMCs with 4 μM OD78 for 24 h did not cause obvious cytotoxicity in serum-free medium (Fig. 2C).

### Effect of OD78 on cell cycle progression in RASMCs induced by PDGF-BB

Flow cytometry (Fig. 3) demonstrated that OD78 affected the cell cycle progression induced by 25 ng/ml PDGF-BB. A primary culture of RASMCs was serum-deprived for 24 h, causing −89.2% of cells to be synthronized in the $G_0/G_1$ phase. Treatment with 1, 2, or 4 μM OD78 reduced the percentage of PDGF-BB-stimulated cells in S phase to 32.8, 59.6, and 87.1%, respectively. Because it is effective against DNA synthesis, OD78 must therefore act during early events in the cell cycle. OD78 arrested significant numbers of cells in the $G_1$ phase, suggesting that its anti-proliferative effects in RASMCs are due to cell cycle arrest.

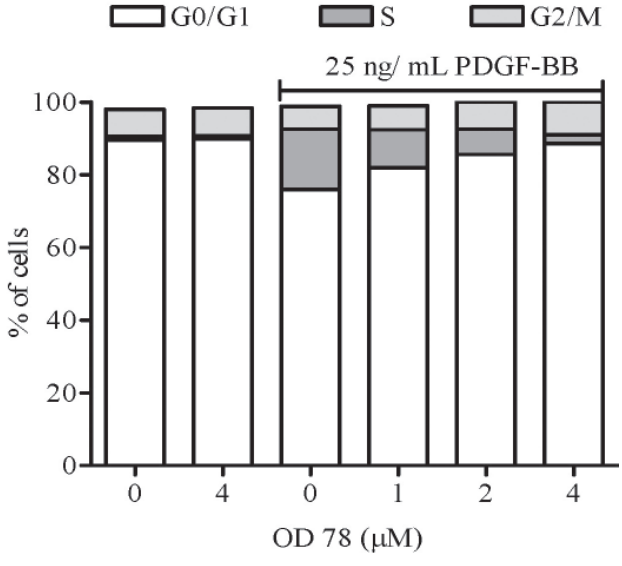


**Fig. 3.** Effect of OD78 on PDGF-BB-induced cell cycle progression. RASMCs were precultured in the presence or absence of OD78 (1, 2, or 4 μM) in serum-depleted medium for 24 h. RASMCs were then stimulated with 25 ng/ml PDGF-BB for 24 h, harvested by trypsinization, and treated as described in the Materials and Methods section. The individual nuclear DNA content was measured as the fluorescence intensity of incorporated PI. Each item is derived from a representative experiment, where data from at least 10,000 events were obtained. Data are representative of at least three independent experiments with similar results.

### Effect of OD78 on PDGF-BB-induced PDGF-Rβ, ERK1/2, Akt, and PLCγ1 phosphorylation

After preincubation with OD78 for 24 h, RASMCs were stimulated with PDGF-BB for 1 min and showed a marked phosphorylation of PDGF-Rβ. Treatment with 1, 2, or 4 μM OD78 had no effect on PDGF-Rβ phosphorylation (Fig. 4A). To determine the effects of OD78 on the downstream signal transduction pathway of PDGF-BB, we assessed the phosphorylation of ERK1/2, Akt, and PLCγ1, but found no changes (Fig. 4B). Therefore, early signaling transduction pathways in PDGF-BB-induced RASMCs may not be involved in the OD78 inhibition of RASMCs proliferation.

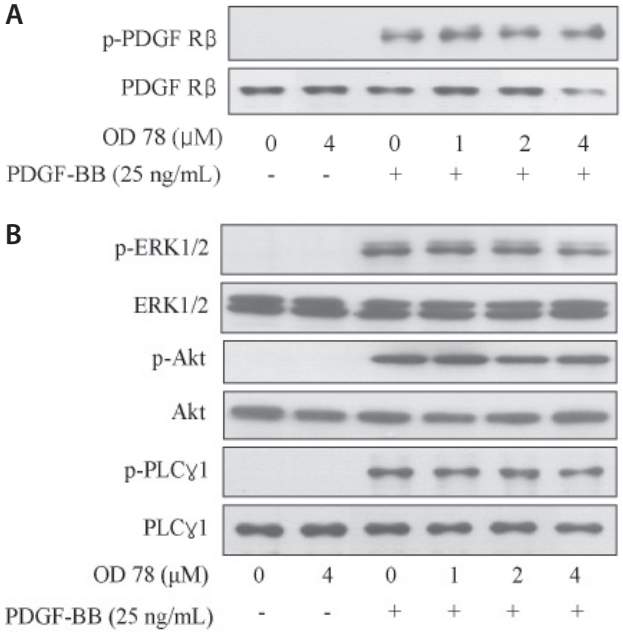


**Fig. 4.** OD78-mediated inhibition of PDGF-BB-induced PDGF-Rβ, ERK1/2, Akt, and PLCγ1 phosphorylation. Confluent cells were precultured in the presence or absence of 1, 2, or 4 μM OD78 in serum-free medium for 24 h. (A) RASMCs were stimulated with 25 ng/ml PDGF-BB for 1 min, lysed, and proteins were analyzed using 7.5% SDS-PAGE and immunoblotting. Total PDGF-Rβ was used for normalization. After densitometric quantification, data were expressed as the mean ± S.E.M. (n=3). (B) OD78-treated cells were stimulated with 25 ng/ml PDGF-BB for 5 min (for ERK1/2 and PLCγ1) or for 15 min (for Akt) at 37°C. The cells were lysed, and the proteins were separated with 10% SDS-PAGE and detected by immunoblotting. Representative data from three independent experiments are presented. The total ERK1/2, Akt, and PLCγ1 were used for normalization.

### Effect of OD78 on PDGF-BB-induced cyclin D1, cyclin E, CDK2, CDK4, and PCNA expression and pRb phosphorylation

Cell cycle progression is tightly regulated through a complex network of positive and negative cell cycle regulatory molecules such as CDKs and cyclins. To characterize the mechanism of OD78-induced cell cycle arrest, the effects of OD78 on cell cycle events such as cyclin D1, cyclin E, CDK2, and CDK4 expression were determined. OD78 significantly decreased the expression of cyclin D1, cyclin E, and CDK4, but had no effect on CDK2 expression.

Rb protein is a key component of the molecular network controlling the cell cycle restriction point. Hypophosphorylated pRb binds to the E2F family of transcription factors, inhibiting transcription of the E2F-responsive genes necessary for cell cycle progression. Pretreatment of OD78 at doses of 1, 2 and 4 μM significantly inhibited the phosphorylation of Rb by 27.6 37.97 and 85.97%, respectively. The PCNA expression was similarly inhibited by OD78 (Fig. 5).

### Inhibitory effect of OD78 on $p21^{cip1}$ and $p27^{kip1}$ expression levels

The effects of OD78 on the expression levels of CKI $p21^{cip1}$ and $p27^{kip1}$ were determined. The expression of $p27^{kip1}$ was rapidly downregulated by PDGF-BB, falling to an undetectable level at 24 h. This decreased $p27^{kip1}$ level of PDGF-BB-induced RASMCs was increased by OD78 treatment (Fig. 6), indicating that the inhibition of Rb protein phosphorylation may occur

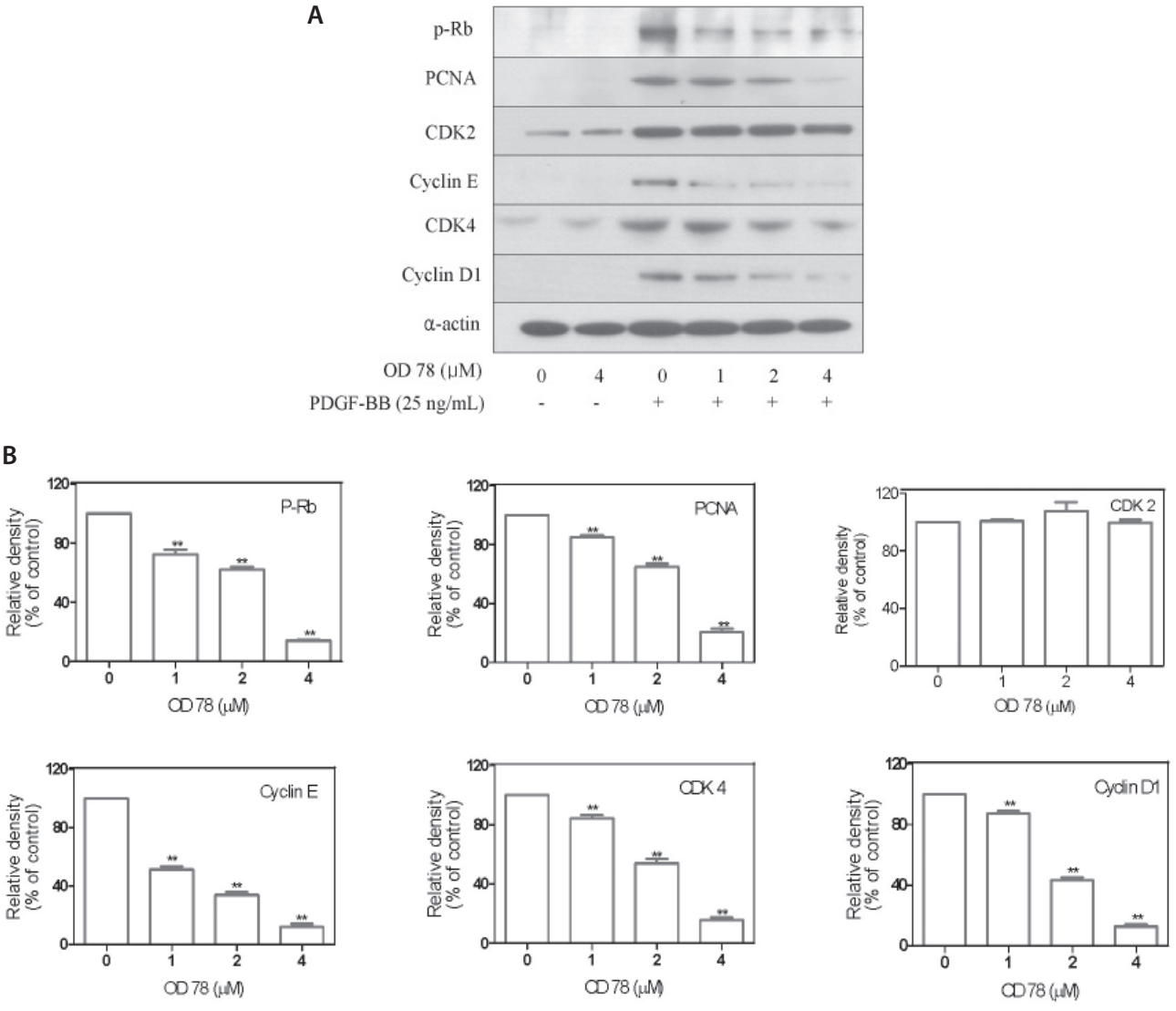


**Fig. 5.** Inhibitory effect of OD78 on PDGF-BB-stimulated expression of cyclin D1, cyclin E, CDK2, CDK4, and PCNA and pRb phosphorylation. Quiescent RASMCs were stimulated for 24 h with 25 ng/ml PDGF-BB in the absence or presence of 1, 2, or 4 μM OD78. The cells were lysed, and proteins were analyzed using 10% SDS-PAGE. Western blot analysis was performed with antibodies specific for cyclin D1, cyclin E, CDK2, CDK4, PCNA, and phopho-Rb. Protein levels were normalized by α-actin. (A) Representative data from three independent experiments. (B) After densitometric quantification, the data were expressed as the mean ± S.E.M. ***p*<0.01 vs. RASMCs stimulated with PDGF-BB alone.

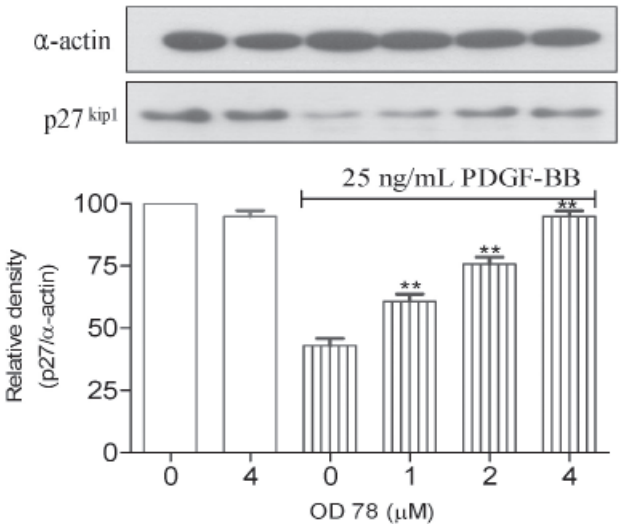


**Fig. 6.** Effect of OD78 on PDGF-BB-stimulated expression of CKIs $p27^{kip1}$. Quiescent RASMCs were stimulated for 24 h with 25 ng/ml PDGF-BB in the absence or presence of 1, 2, or 4 μM OD78. The cells were lysed, and the proteins were separated with 12.5% SDS-PAGE and immunoblotting. Protein levels were normalized by α-actin. After densitometric quantification, the data were expressed as the mean ± S.E.M. $^{**}p<0.01$ vs. RASMCs stimulated with PDGF-BB alone.

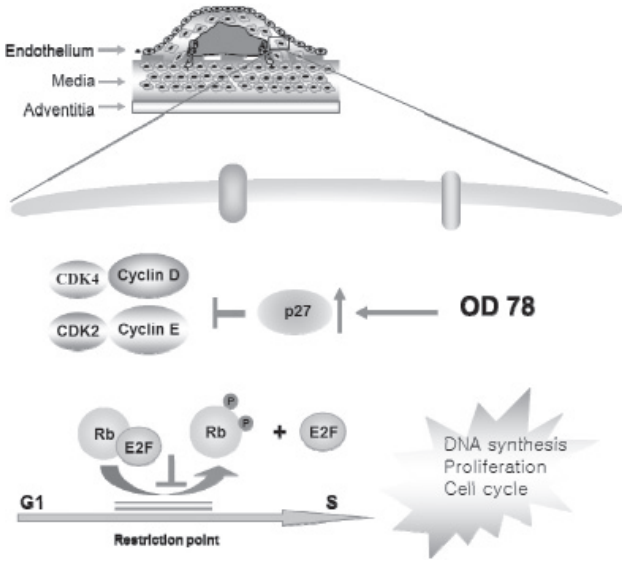


**Fig. 7.** Schematic diagram for the cell cycle regulation mechanism of OD78. Target proteins for OD78 in the PDGF-BB signaling pathway in RASMCs did not include phosphorylation of PDGF-Rβ or early signals such as ERK1/2, PLCγ1, and Akt. However, OD78 appeared to inhibit the cell cycle progression from $G_0/G_1$ to S phase. OD78 upregulates p27, thereby inhibiting cyclin D, cyclin E, and CDK 4 expression, followed by the decreased expression of PCNA and the reduced phosphorylation of Rb. Thus, OD78 exposed to RASMCs inhibits cell cycle progression.

by blocking CDK activities following OD78-mediated $p27^{kip1}$ activation. The $p21^{cip1}$ level was decreased by the addition of PDGF-BB, but OD78 did not cause any change in $p21^{cip1}$ expression. Thus, $p21^{cip1}$ does not appear to be involved in the anti-proliferative effect of OD78 on RASMCs (data not shown).

## DISCUSSION

The proliferation of VSMCs in response to vessel injury is associated with several pathophysiological conditions, such as inflammation, pulmonary hypertension, and coronary artery restenosis following balloon angioplasty, eventually leading to cardiovascular disease (Ross, 1993). Therefore, the inhibition of VSMC proliferation is important for developing cardiovascular disease therapies. In the present study, we investigated the anti-proliferative activity of OD78, a newly synthesized obovatol derivative, on PDGF-BB-induced RASMCs and the related signal transduction pathway in cultured RASMCs.

VSMC proliferation within the intimal layer of the vessel wall is a key step in atherosclerotic plaque development. PDGF, one of the most potent mitogenic and chemotactic agents for VSMCs, is released by platelets, endothelial cells, and VSMCs themselves and is a major component of FBS (Stroobant and Waterfield, 1984). In this study, OD78 potently inhibited the RASMC proliferation (Fig. 2A) and DNA synthesis (Fig. 2B) induced by PDGF-BB. The anti-proliferative effect of OD78 was not due to cytoxicity (Fig. 2C), but was associated with cell cycle arrest in the $G_0/G_1$ phase (Fig. 3). OD78 had no effect on the PDGF-BB-stimulated phosphorylation of PDGF-Rβ (Fig. 4A), ERK1/2, PLCγ1, or Akt (Fig. 4B). This result suggests that the OD78-mediated inhibition of RASMC proliferation may not occur at the receptor level and may not involve PDGF-BB-mediated early signals such as ERK1/2, PLCγ1, and Akt.

In the mammalian cell cycle, cell division and growth are tightly controlled by a series of positive and negative regulators that act at sequential points throughout the cell cycle. VSMCs are stimulated to divide in response to mitogens after a vascular injury, resulting in their exit from the $G_1$ phase and entry into the S phase (Ross, 1995). The $G_1$ phase is the major control point for mammalian cell proliferation (Fang and Newport, 1991). We found that the inhibitory effect of OD78 on RASMCs was exerted during the $G_1$ phase, and specifically investigated proteins that regulate the cell cycle.

CDKs play a central role in cell cycle regulation and promote the G1/S transition by phosphorylating Rb protein (Weinberg, 1995; Sriram and Patterson, 2001). CDK2 and CDK4 are key mediators for the $G_0/G_1$ to S phase progression by forming complexes with cyclin D1 and E (Jirawatnotai *et al.*, 2004; Martin *et al.*, 2005). These complexes lead to the hyperphosphorylation of Rb and thereby promote DNA synthesis (Sherr, 1996). We observed that OD78 inhibited cyclin D1, cyclin E, and CDK4 expression and suppressed Rb protein phosphorylation, but had no effect on CDK2 expression (Fig. 5). Several CDKs are known to phosphorylate pRb (Akiyama *et al.*, 1992; Connell-Crowley *et al.*, 1998). The inhibition of CDK4, cyclins D1 and E, and Rb protein phosphorylation may be sufficient, and inhibition of CDK2 may not be necessary, to arrest cell cycle progression (Sasaguri *et al.*, 1996; Brooks *et al.*, 1997). The expression of PCNA, a phospho-Rb-mediated gene product in the early G0/G1 and S phases of the cell cycle, was also inhibited by OD78.

CDK inhibitor proteins can be classified into the Cip/Kip (e.g., p21, p27 and p57) and INK4 families. The Cip/Kip family inhibits CDK2, CDK4 and CDK6 expression. p27 expression can be regulated by growth factors for cell cycle progres-

sion. Upon stimulation of PDGF-BB, the level of p27 protein in VSMCs is reduced (Servant *et al.*, 2000). We observed that OD78 inhibited the downregulation of $p27^{kip1}$ (Fig. 6), but not $p21^{cip1}$ (data not shown), after PDGF-BB stimulation. $p27^{kip1}$ reportedly abrogates the activities of both CDK2 and CDK4 as well as VSMC proliferation *in vitro* and *in vivo* after balloon-injury in pig (Tanner *et al.*, 2000). Some drugs that upregulate $p27^{kip1}$ expression reportedly exhibit potent anti-proliferative activities on VSMCs (Chen *et al.*, 1997; Marra *et al.*, 2000). Thus, our finding that OD78 selectively upregulates $p27^{kip1}$ expression is consistent with its greater inhibitory effects on the expression of multiple cell cycle proteins.

The obovatol derivative JY0691 potently inhibits PDGF-BB-induced RASMC proliferation and arrests cell cycle progression at $G_0/G_1$ through $p21^{cip1}$ (Yu *et al.*, 2009). The present study reveals that OD78 also inhibits PDGF-BB-induced RASMC proliferation via $G_0/G_1$ arrest, in association with Rb protein, cyclin D1, cyclin E, CDK4, and PCNA downregulation and $p27^{kip1}$ upregulation (Fig. 7). Therefore, $p27^{kip1}$ may serve as a potential target for the OD78-induced inhibition of cell proliferation. The anti-proliferative property of OD78 may be useful for the treatment of patients with atherothrombosis or at a high risk for cardiovascular disease.

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