BIOCHEMICAL PHARMACOLOGY 75 (2008) 1331–1340

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JM91, a newly synthesized indoledione derivative, inhibits rat aortic vascular smooth muscle cells proliferation and cell cycle progression through inhibition of ERK1/2 and Akt activations

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ARTICLE INFO

Article history:
Received 7 September 2007
Accepted 28 November 2007

Keywords:
Indoledione
Vascular smooth muscle cell
Platelet-derived growth factor
Phosphatidylinositol 3-kinase
Extracellular signal-regulated kinase

ABSTRACT

The increased potential for growth of vascular smooth muscle cells (VSMCs) is a key abnormality in the development of atherosclerosis and postangioplasty restenosis. Platelet-derived growth factor (PDGF)-BB is a potent mitogen for VSMCs that plays an important role in the intimal accumulation of VSMCs. This study examined the effect of JM91, a newly synthesized indoledione derivative, on the proliferation of PDGF-BB-stimulated rat aortic VSMCs. The antiproliferative effect of JM91 on rat aortic VSMCs was examined by cell counting and [^{3}H]thymidine incorporation assay. The pre-incubation of JM91 (0.5–3.0 μM) significantly inhibited the proliferation and DNA synthesis of 25 ng/mL PDGF-BB-stimulated rat aortic VSMCs in a concentration-dependent manner. JM91 inhibited the PDGF-BB-stimulated phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) and Akt kinase, while had no effect on PLCγ1 and PDGF-Rβ activation. In addition, treatment with JM91 (0.5–3.0 μM) induced cell-cycle arrest in the G_1 phase, which was associated with the down–regulation of cyclins and CDKs. These findings suggest that the inhibitory effects of JM91 against proliferation, DNA synthesis and cell cycle progression of PDGF-BB-stimulated rat aortic VSMCs are mediated by the suppression of the ERK1/2 and PI3K/Akt signaling pathways. Furthermore, JM91 may be a potential antiproliferative agent for the treatment of atherosclerosis and angioplasty restenosis.

1. Introduction

Atherosclerosis is one of the causes of a variety of cardiovascular diseases, and there is evidence suggesting that it is a chronic inflammatory disease state [1,2]. The primary event in the development of atherosclerosis and restenosis is believed to involve an injury to the endothelium, leading to a similar response to wound healing, which requires the migration of vascular smooth muscle cells (VSMCs) from the media to the intimal with subsequent proliferation [3–5]. Although various

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doi:10.1016/j.bcp.2007.11.013

growth factors and cytokines are involved in the proliferation of VSMCs, one of the principal regulators of mitogenesis in VSMCs is platelet-derived growth factor (PDGF) [6]. PDGF is produced by activated macrophages, VSMCs and endothelial cells, forming three isoforms (AA, AB, and BB). PDGF-BB is a considerably more potent proliferative stimulus to VSMCs than PDGF-AA [7]. The PDGF-BB-stimulated mitogenesis signaling pathway has been well characterized. The binding of PDGF-BB to the PDGF-receptor (PDGF-R) can activate three major signal transduction pathways; phosphatidylinositol 3-kinase (PI3K), phospholipase C (PLC)γ1 and extracellular regulated kinases 1/2 (ERK1/2) by activating Raf-1 [8]. It was reported that ERK1/2 activation is essential for its mitogenic signaling through a number of tyrosine kinase growth factor receptors, and is associated with the development and progression of proliferative cardiovascular diseases, such as hypertension and atherosclerosis [8–11].

Mammalian cell proliferation is governed by the cell cycle [12], which is a complex, stepwise process that involves the resting G_0 phase, and cell growth involving the G_1, S and G_2/M phases. VSMCs are normally quiescent in arterial media, and proliferate at low indices (<0.05%), remaining in the G_0/G_1 phase of the cell cycle [13]. The kinase activity of cyclin-dependent kinases (CDKs) is governed by regulatory subunits known as cyclins. These form a complex with their catalytic subunit CDKs and are regulated at a specific phase of the cell cycle [14–16]. In many cells, transit through the G_1 phase of the cell cycle and entry into the S phase require the binding and activation of cyclin/CDK complexes, mainly cyclin D_1/CDK 4 and cyclin E/CDK 2 [12].

Indole derivatives have been used as antiviral agents against HIV-1 wt (wild type) and as anticancer agents [17–20]. However, the antiproliferative effect of indole or indoledione derivatives on VSMCs is unknown. The aim of this study was to elucidate the antiproliferative effect and molecular mechanism of JM91, a newly synthesized indoledione derivative, on PDGF-BB-stimulated rat aortic VSMCs. The results suggest that JM91 potently inhibits the proliferation of VSMCs by suppressing the ERK1/2 and PI3K/Akt signaling pathways stimulated by PDGF-BB in VSMCs. Moreover, JM91 arrests cell cycle progression in the G_0/G_1 phase by downregulating the expression of cyclin D_1, cyclin E, CDK4 and CDK2 proteins as well as retinoblastoma (Rb) protein phosphorylation.

2. Materials and methods

2.1. Chemicals and reagents

The cell culture materials and FBS were obtained from Gibco-BRL (Gaithersburg, MD). [^{3}H]thymidine was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). PDGF-BB was acquired from Upstate Biotechnology (Lake Placid, NY). Phospho-ERK1/2, phospho-Akt, phospho-PLCγ1, phospho-PDGF-Rβ, ERK1/2, Akt, PLCγ1, PDGF-Rβ and phospho-pRb antibodies were supplied by Cell Signaling Technology Inc. (Beverly, MA). PDGF-Rβ and phospho-PDGF-Rβ antibodies were obtained from Upstate Biotechnology (NY). Cyclin D_1, cyclin E, CDK2 and CDK4 antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The other chemicals used were of the highest analytical grade commercially available.

2.2. Synthesis of JM91

All melting points were measured using Büchi melting point B-545 and were uncorrected. The ^{1}H NMR spectra were recorded on a Varian Unity INOVA 400 MHz FT-NMR spectrometer using $DMSO_{-d6}$ or $CDCl_3$ with TMS. The mass spectra were obtained using a Jeol JMS AX505 WA. The reagents were purchased from Aldrich Chemical Co. The JM91 (methyl 2-amino-4,9-dihydro-4,9-dioxo-1-*p*-tolyl-1H-pyrrolo[3,2-g]isoquinoline-3-carboxylate) was prepared by cyclizing 6,7-dichloroisoquinoline-5,8-dione with methyl cyanoacetate and arylyamine according to the method reported elsewhere [21].

JM91 (Fig. 1A): mp 219–220 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.48(s, 3H, methyl), 3.96(s, 3H, methoxy), 6.97(m, 2H, benzene), 7.04(m, 2H, benzene), 7.50(s, 2H, NH2), 7.83(d, 1H, J = 5.2, 3-pyridine), 9.07(d, 1H, J = 5.2, 3-pyridine), 9.44(s, 1H, 3-pyridine); MS (*m*/*z*) 361 (M^+).

2.3. Cell culture

Rat aortic vascular smooth muscle cells (RAVSMCs) were isolated by enzymatic dispersion using a slight modification of the method reported by Chamley et al. [22]. As previously described [23], the cells were cultured in DMEM supplement with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 8 mM HEPES and 2 mM L-glutamine at 37 °C in a humidified 5% CO_2 incubator. The purity of the RAVSMCs cultures was >95%, as confirmed by immunocytochemical staining of α-smooth muscle actin. Passages 5–9 of the RAVSMCs were used in these experiments.

2.4. Measurement of cell proliferation and DNA synthesis

The level of RAVSMCs proliferation was measured by direct cell counting. Briefly, the cells were seeded at a concentration of 4×10^4 cells/well in a 12-well culture plate and grown in DMEM containing 10% FBS for 24 h. The cells were then cultured with serum free (0.4% FBS) medium containing JM91 for 24 h. The cells were treated with 25 ng/mL PDGF-BB and counted 24 h later using hemocytometer. The effects of JM91 on cell proliferation were examined. DNA synthesis was assayed by measuring the level of [^{3}H]thymidine incorporation into the cell DNA [24]. The RAVSMCs were seeded in 24-well culture plates at a concentration of 7000 cells/well and grown until they had reached 60% confluence. The medium was then replaced with serum-free (0.4% FBS), which consisted of DMEM containing various concentrations of JM91. After 24 h, the cultures were exposed to 25 ng/mL PDGF-BB for 20 h before adding 1 μCi/mL [^{3}H]thymidine to the medium. Four hours later, the labeling reactions were quenched by aspirating the medium and subjecting the cultures to sequential washes on ice with PBS containing 10% trichloroacetic acid and ethanol/ether (1:1, v/v). The acid-insoluble [^{3}H]thymidine was extracted into 500 μL of 0.5 M NaOH/well. This solution was mixed with 3 mL of scintillation cocktail (UltimaGold, Packard Bioscience, CT, USA) and quantified using a liquid scintillation counter (LS3801, Beckman, Düsseldorf, Germany).

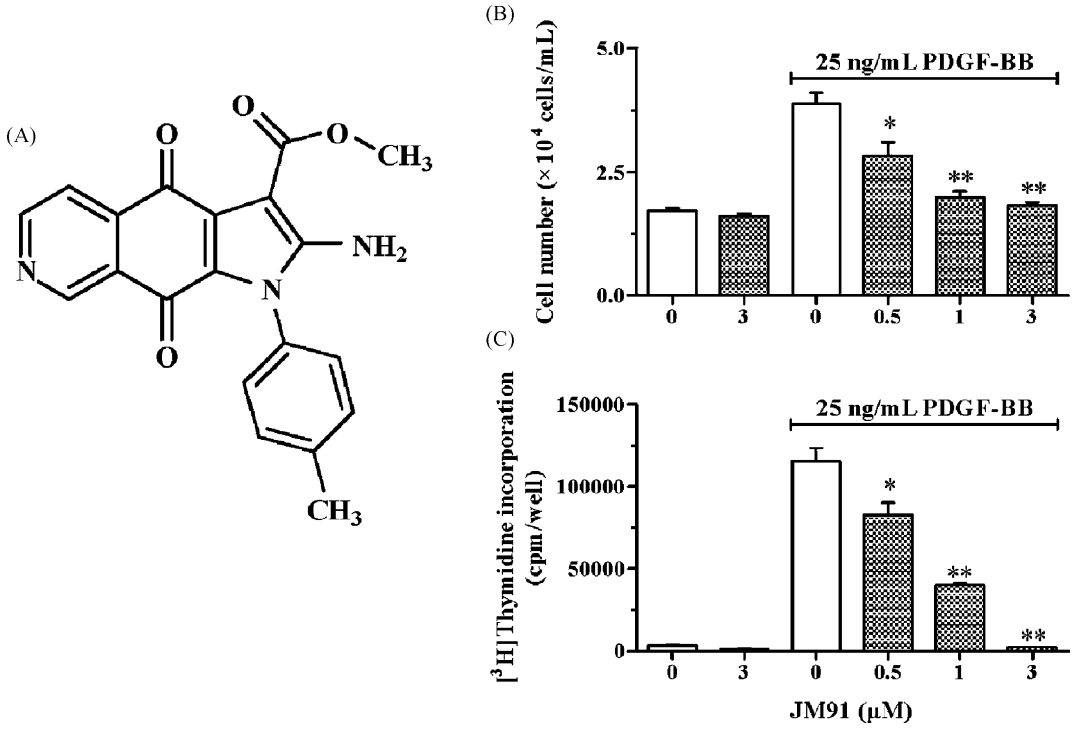

Fig. 1 – Effect of JM91 on PDGF-BB-stimulated proliferation and DNA synthesis of rat aortic VSMCs. (A) Chemical structure of methyl 2-amino-4,9-dihydro-4,9-dioxo-1-*p*-tolyl-1H-pyrrolo[3,2-g]isoquinoline-3-carboxylate (JM91). (B) Effect of JM91 on the number of PDGF-BB-stimulated RAVSMCs. The RAVSMCs were pre-cultured in the serum-free medium in the presence or absence of JM91 (0.5–3.0 μM) for 24 h, and then stimulated by 25 ng/mL PDGF-BB for 24 h. The cells were trypsinized, and counted using a hemocytometer. (C) The RAVSMCs were cultured in serum-starved medium in the presence or absence of JM91 (0.5–3.0 μM) for 24 h, and exposed to 25 ng/mL PDGF-BB for 20 h before 1 μCi/mL [^{3}H]thymidine was added to the medium. The labeling reaction was quenched and quantified using a liquid scintillation counter, 4 h later. The data is reported as the mean ± S.E.M. from four different set of experiments. *P < 0.05 and **P < 0.01 vs. only PDGF-BB-stimulated RAVSMCs.

2.5. *Cell cycle analysis*

The proportions of cells in various phases of the cell cycle were estimated by measuring cellular DNA content by flow cytometry (FACS). The cells were harvested, fixed in 70% ethanol, and stored at −20 °C. The cells were then washed twice with ice-cold PBS and incubated with RNase, and the PI-DNA complex in each cell nucleus was measured using FACS Calibur (Becton & Dickinson Co.). The rate of the cell cycle within the G_0/G_1, S and G_2/M phases was determined by analysis with the computer program, Modfir LT (Verity Software House, Inc.).

2.6. *Annexin-V and PI double staining assay*

Both propidium iodide (PI) and annexin-V labeling for apoptotic or necrotic cell death detection was carried out using an Annexin-V-FLUOS staining kit according to the manufacturer's instructions (Roche, Germany). Annexin-V is a protein that binds to phosphatidylserine residues, which are exposed on the surface of apoptotic cells but not on normal cells [25]. Briefly, rat aortic VSMCs in a 6 well plate were pre-incubated in serum-free medium containing a mixture DMEM medium (Gibco-BRL, USA) in the presence and absence of various concentrations of JM91 for 24 h. The rat aortic VSMCs were then stimulated with 25 ng/mL of PDGF-BB for 24 h. The cells were then trypsinized, collected, rinsed twice with cold PBS, resuspended in 100 μL of a binding buffer, and incubated with 2 μL FITC-labeled annexin-V and 2 μL propidium iodide (PI, stock solution 50 μg/mL) for 15–25 min in the dark at room temperature. The rat aortic VSMCs were analyzed by FACS Calibur (Becton & Dickinson Co., USA). After the appropriate markings for the negative and positive populations were set, the percentage of annexin-V−/PI− (living cells), annexin-V+/PI− (apoptotic cells), annexin-V+/PI+ (necrotic cells) staining were determined.

2.7. *Western blotting*

The cell lysates were separated on SDS-PAGE containing 10–15% acrylamide gels according to the method described by Laemmli [26]. As previously described [27], the proteins were transferred to PVDF membranes (Millipore Corp.), which were then blocked overnight at 4 °C in Tris-buffered saline containing 0.1% Tween 20 (TBS/T) and 5% bovine serum albumin (BSA). The membranes were then incubated with a 1:1000 dilution of phospho-ERK1/2, phospho-Akt, phospho-PLCγ1, ERK1/2, Akt, PLCγ1, CDK2, CDK4, cyclin D_1, cyclin E and phospho-pRb antibodies. The blots were washed with TBS/T, and then incubated with a 1:5000 dilution of horseradish peroxidase-conjugated IgG secondary antibody (New England Biolabs, MA, USA). The proteins were detected using a chemiluminescent reaction (ECL plus kit, Amersham Phar-

macia Biotech) followed by exposure of the membranes to Hyperfilm ECL (Amersham Pharmacia Biotech, Buckinghamshire, UK). The band intensities were quantified using Scion-Image® Software for windows®.

2.8. Statistical analysis

The experimental results are expressed as the mean ± S.E.M. One-way analysis of variance (ANOVA) was used for multiple comparison followed by a Dunnett's test. P values <0.05 and <0.01 were considered significant.

3. Results

3.1. Effect of JM91 on PDGF-BB- stimulated rat aortic VSMCs proliferation and DNA synthesis

The effect of JM91 was assessed by direct cell counting to determine if JM91 inhibits PDGF-BB-stimulated RAVSMCs proliferation. A treatment with 25 ng/mL PDGF-BB for 24 h resulted in a significant increase in the number of cells, which was decreased significantly by pre-treatment of JM91 in a concentration-dependent manner. The percentage of inhibition at concentrations of 0.5, 1.0 and 3.0 μM was 48.7 ± 2.3%, 87.2 ± 0.8% ($P < 0.01$) and 94.9 ± 0.6% ($P < 0.01$), respectively (Fig. 1B). The level of [^{3}H]thymidine incorporation into the DNA was also measured as an index of cell proliferation. In the absence of PDGF-BB, the RAVSMCs incorporated [^{3}H]thymidine into the DNA but stimulation with 25 ng/mL PDGF-BB caused a significant increase in the level of [^{3}H]thymidine incorporation. As shown in Fig. 1C, JM91 inhibited the PDGF-BB-stimulated DNA synthesis in a concentration-dependent manner, with inhibitory percentages of 28.9, 67.1 and 100% at concentrations of 0.5, 1.0 and 3.0 μM, respectively (Fig. 1C).

3.2. Effect of JM91 on cell cycle progression

As shown in Fig. 2, the serum-deprivation of RAVSMCs in the primary culture for 24 h resulted in an approximately 85.5%

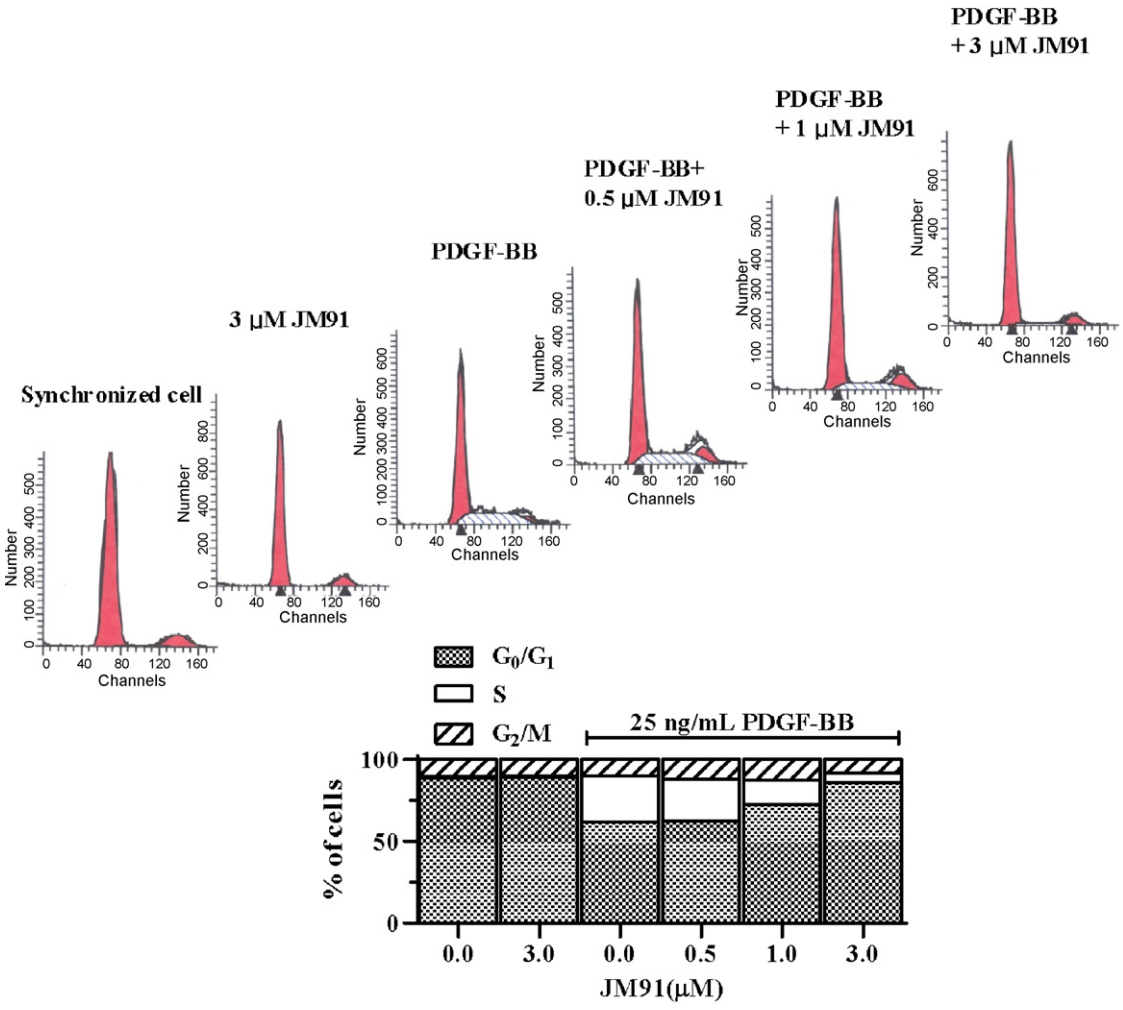

Fig. 2 – Effect of JM91 on PDGF-BB-stimulated cell cycle progression. The cells were pre-cultured in the presence or absence of JM91 in serum-depleted medium for 24 h. The cells were stimulated with 25 ng/mL of PDGF-BB. After 24 h, the cells were harvested by trypsinization and analyzed by flow cytometry. Each item in up panel is derived from a representative experiment, where data from at least 10,000 events were obtained. The data are expressed as Mean values from three independent experiments in low panel.

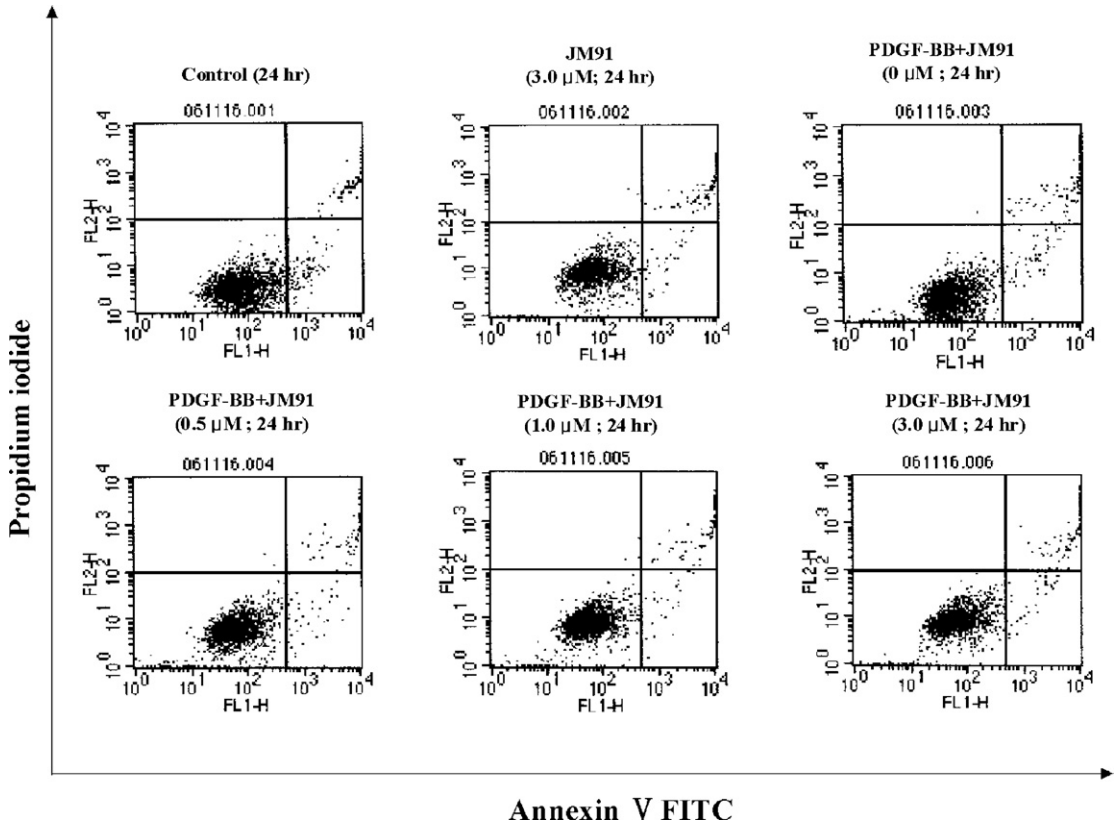

Fig. 3 – Flow cytometry dot plots of rat aortic VSMCs at 24 h after PDGF-BB-stimulation in the presence of JM91. The cells were double-stained with annexin V (shown on the *x*-axis, logarithmic scale) and propidium iodide (shown on the *y*-axis, logarithmic scale) at 24 h after 25 ng/mL PDGF-BB-stimulation in the presence or absence of various concentrations of JM91 (0.5, 1 and 3 μM). The cells positive for annexin V only (early apoptosis) were found in the lower right panel, and the cells positive for annexin V and propidium iodide (late apoptosis/necrosis) are shown in the upper right panel. The results are representative of three independent experiments.

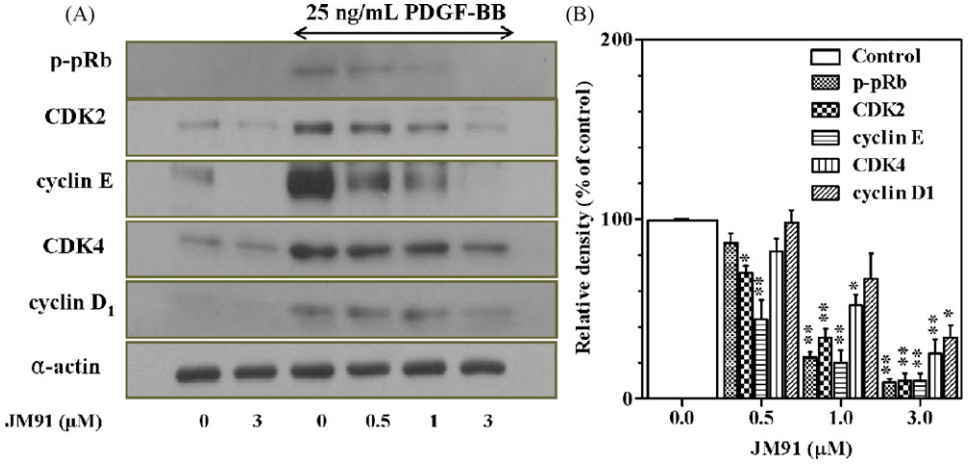

Fig. 4 – Effects of JM91 on PDGF-BB-stimulated CDK2, CDK4, cyclin D_1 and cyclin E expressions and pRb phosphorylation. Quiescent RAVSMCs were stimulated with 25 ng/mL PDGF-BB either in the presence or absence of various concentrations of JM91 for 24 h. The cells were then lysed, and proteins were analyzed using 12% SDS-PAGE. Western blot analysis was performed with the antibodies specific for CDK2, CDK4, cyclin D_1, cyclin E and phospho-pRb. α-Actin was used for normalization. (A) Representative data from three different experiments are presented. (B) After densitometric quantification, the data is expressed as the mean ± S.E.M. ($n = 3$). $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. only PDGF-BB-stimulated RAVSMCs.

synchronization of the cell cycle in the G_0/G_1 phase. The percentage of cells in the S phase increased from 2.6 ± 1.3 to 23.4 ± 1.0% for 24 h after serum repletion. In contrast, cell cycle progression was blocked significantly in the JM91-treated cells. The reduced-percentage of cells in the S phase was 14.6 ± 4.7% ($P < 0.05$, $n = 5$, duplicate), 13.2 ± 1.5% and 6.9 ± 2.3% ($P < 0.01$, $n = 5$, duplicate) at concentrations of 0.5, 1.0 and 3.0 μM, respectively, indicating that JM91 might act at the early events of the cell cycle to be effective against DNA synthesis.

3.3. Effect of JM91 on viability of rat aortic VSMCs

The apoptotic cells were double-stained with annexin V (shown on the *x*-axis, logarithmic scale) and propidium iodide (shown on the *y*-axis, logarithmic scale) 24 h after stimulation with PDGF-BB in the presence or absence of various concentrations of JM91 (0.5, 1 and 3 μM). As shown in Fig. 3, the antiproliferative effect of JM91 was not due to the induction of VSMCs apoptosis as there were no significant proportions apoptotic or necrotic cells detected in the PDGF-BB-stimulated RAVSMCs in the presence of various concentrations of JM91.

3.4. Effect of JM91 on cell cycle regulatory protein expression

The JM91-treatment results in G_0/G_1 phase cell cycle arrest. Therefore, this study examined the effect of JM91 on cell cycle regulatory molecules related to the G_1 phase of the cell cycle, which involve the sequential activation of certain serine/threonine protein kinases known as CDKs that phosphorylate the Rb protein. As shown in Fig. 4, after 24 h stimulation with PDGF-BB, there was an increase in the expression of cyclin D_1 and cyclin E as well as CDK2 and CDK4, while treatment of JM91 (0.5–3.0 μM) resulted in a concentration-dependent inhibitions. Accordingly, JM91 inhibited the PDGF-BB-induced pRb hyperphosphorylation in a similar concentration-dependent manner as it suppressed cell proliferation, cell cycle progression and DNA synthesis.

3.5. Effect of JM91 on PDGF-BB-induced PDGF-Rβ phosphorylation

After pre-incubating the RAVSMCs with various concentrations of JM91 for 24 h, the RAVSMCs were stimulated for 1 min with PDGF-BB, which caused the marked phosphorylation of PDGF-Rβ. However, treating the RAVSMCs with JM91 had no significant effect on PDGF-Rβ phosphorylation (Fig. 5).

3.6. Effect of JM91 on ERK1/2, PI3K/Akt and PLCγ1 phosphorylation

The signaling pathway in PDGF-BB-stimulated mitogenesis was relatively well characterized [27]. In particular, PDGF-BB binding to the PDGF receptor leads to the activation of three major signal transduction pathways: mitogen-activated protein (MAP) kinase, PI3K/Akt, and PLCγ1. Therefore, it was hypothesized that the inhibition induced by JM91might involve the ERK1/2, PI3K/Akt and PLCγ1 pathways. The

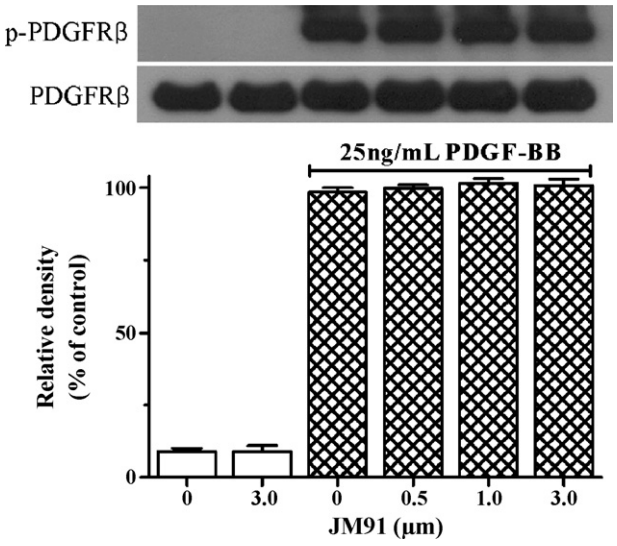

Fig. 5 – Effect of JM91 on PDGF-BB-induced PDGF-Rβ phosphorylation in rat aortic VSMCs. Confluent cells were pre-cultured in the presence or absence of JM91 (0.5–3 μM) in serum-free medium for 24 h, and stimulated by 25 ng/mL PDGF-BB at 37 °C for 1 min. The cells were lysed, and proteins were analyzed using 7.5% SDS-PAGE and immunoblotting. The total α-actin was used for normalization. After densitometric quantification, the data is expressed as the mean ± S.E.M. ($n = 3$).

RAVSMCs were pre-cultured in the presence or absence of JM91 (0.5–3.0 μM) in a serum-free medium for 24 h, and then stimulated for 1–30 min with 25 ng/mL PDGF-BB. As shown in Figs. 6 and 7, 25 ng/mL of PDGF-BB stimulated the maximum ERK1/2, PI3K/Akt and PLCγ1 phosphorylation at 5, 15 and 5 min, respectively. The increased phosphorylation of ERK1/2 and PI3K/Akt were significantly inhibited by JM91 in a concentration-dependent manner, while the phosphorylation of PLCγ1 was not affected when PDGF-BB had induced the maximum ERK1/2, PI3K/Akt or PLCγ1 phosphorylation.

3.7. Synergistic effects of JM91 and inhibitors of ERK1/2 and Akt on DNA synthesis

These results show that JM91 inhibits the proliferation of RAVSMCs through the suppression of PDGF-BB-stimulated ERK1/2 and Akt activation. In order to confirm whether cooperatively inhibiting both pathways can reach a synergistic inhibition on cell proliferation, we looked at the synergistic effects of U0126 (an ERK1/2 inhibitor), LY294002 (a PI3K/Akt pathway inhibitor) and JM91 when they were used along or in combination. As shown in Fig. 8, treatments of cells with U0126 (1 μM), LY294002 (2 μM) or JM91 (0.5 μM), respectively, can produce a partial inhibition of DNA synthesis; however, when they used in combination, potent additive/synergistic inhibitory effects were occurred. Therefore, this result clearly indicated that the inhibitory effects of JM91 on ERK1/2 and Akt activation are sufficient to block the PDGF-BB-stimulated proliferation of RAVSMCs.

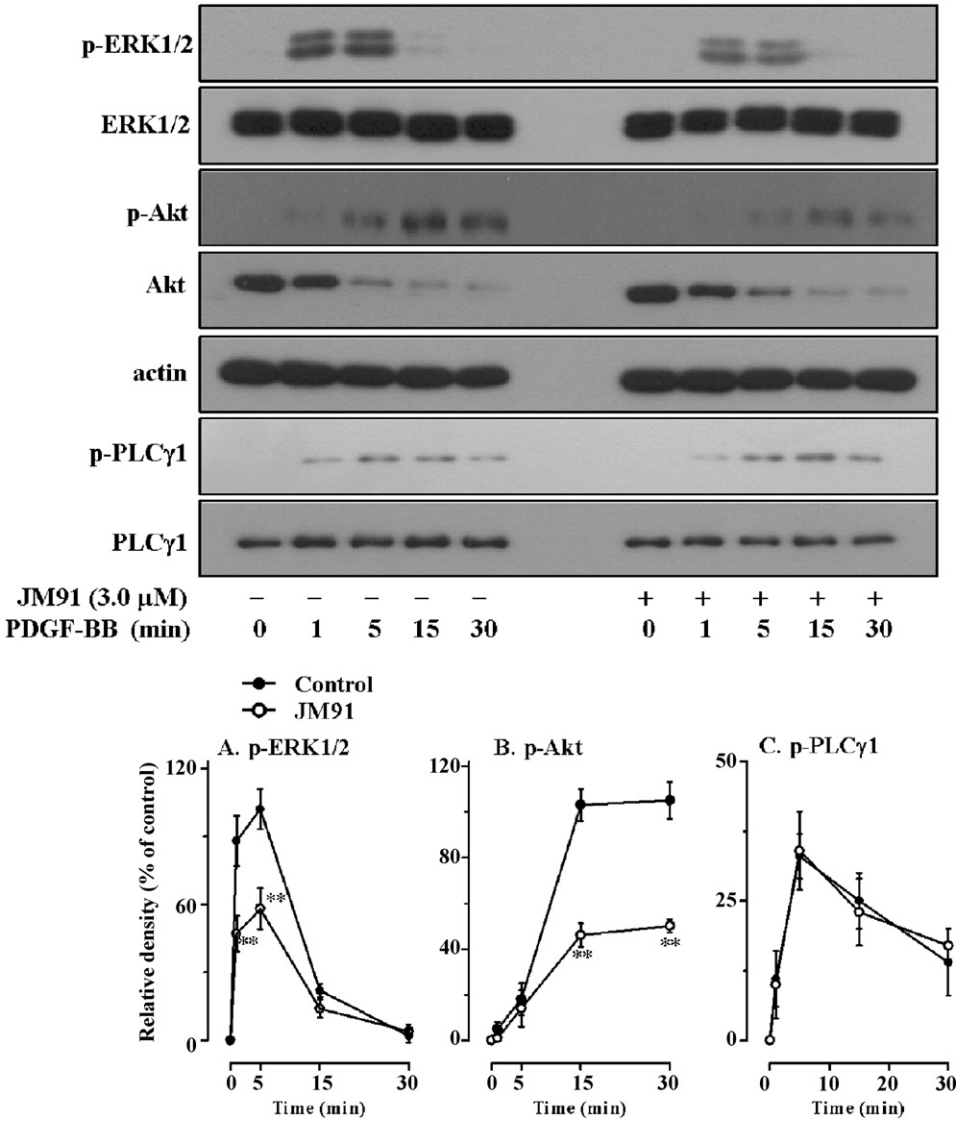

Fig. 6 – Effects of JM91 on time course of ERK1/2, Akt and PLCγ1 phosphorylations in PDGF-BB-stimulated rat aortic VSMCs. Quiescent RAVSMCs were stimulated with 25 ng/mL PDGF-BB either in the absence or presence of 3.0 μM JM91 for the indicated times. The cells were lysed, and the proteins were analyzed using 10% SDS-PAGE. The total protein was used for normalization respectively. (A) phospho-ERK1/2, (B) phospho-Akt, (C) phospho-PLCγ1. *P < 0.05 and **P < 0.01 vs. only PDGF-BB-stimulated RAVSMCs.

4. Discussion

The abnormal migration and proliferation of VSMCs in the arterial walls are important pathogenetic factors of vascular disorders such as atherosclerosis and restenosis after angioplasty [5]. Therefore, inhibiting the proliferation of VSMCs is a potentially important therapeutic strategy for treating these diseases [28]. The aims of this study were to determine if JM91, a newly synthesized indoledione derivate (Fig. 1A), has an antiproliferative effect and to define its mechanisms of action on PDGF-BB-stimulated rat aortic VSMCs.

It was found that JM91 inhibited the proliferation and DNA synthesis of RAVSMCs stimulated with 25 ng/mL PDGF-BB (Fig. 1B and 1C). In addition, the antiproliferative effect of JM91 was associated with cell cycle arrest in the G_0/G_1 phase (Fig. 2) without any induction of apoptosis (Fig. 3). This indicates that JM91 has a specific effect on cell cycle progression rather than decreasing the number of cells through apoptosis. It was recently reported that VSMCs are stimulated to divide in response to mitogens after a vascular injury, which results in their exit from the G_1 phase and entry into the S phase [10]. Some studies report that the G_1 phase is a major point of control for cell proliferation in mammalian cells [29]. Beyond this point, the cells are committed to DNA replication, and further cell cycle progression proceeds independently of growth factor stimulation. The Rb protein is a key component of the molecular network controlling the restriction point, which maintains the hypophosphorylated state to bind the E2F family of transcription factors and inhibits the transcription of E2F-responsive genes essential for cell cycle progression and DNA synthesis. As shown in Fig. 4, a treatment with JM91 resulted in the concentration-dependent inhibition of

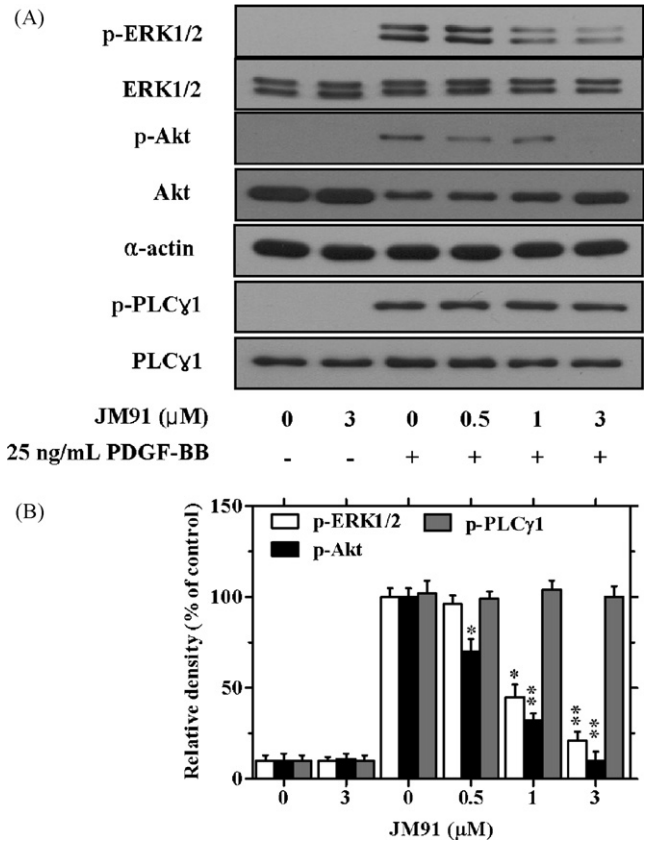

Fig. 7 – Effects of JM91 on the phosphorylations of ERK1/2, Akt and PLCγ1 in PDGF-BB-stimulated rat aortic VSMCs. The quiescent RAVSMCs were pre-incubated with various concentrations of JM91 for 24 h, and stimulated with PDGF-BB for 5, 15 and 5 min for ERK1/2, Akt and PLCγ1 activation, respectively. The cells were then lysed, and the proteins were separated with 10% SDS-PAGE and detected by immunoblotting. (A) Representative data from three different experiments are presented and the total ERK1/2, Akt and PLCγ1 were used for normalization. (B) After densitometric quantification, the data is expressed as the mean ± S.E.M. *P < 0.05 and **P < 0.01 vs. only PDGF-BB-stimulated RAVSMCs.

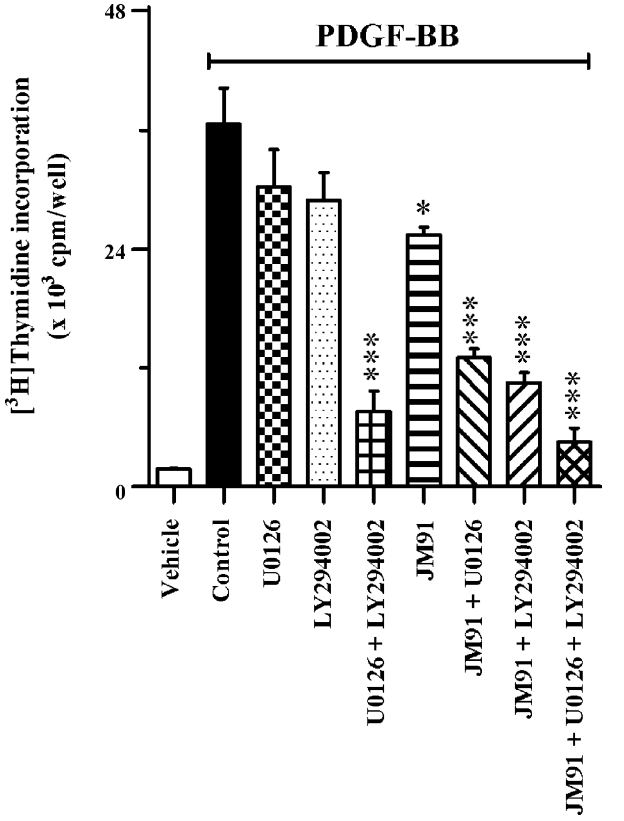

Fig. 8 – Synergistic effects of ERK1/2 and Akt inhibitors, and JM91 on DNA synthesis. Confluent cells were precultured with U0126 (an ERK 1/2 inhibitor, 1 μM), LY294002 (a PI3K/Akt pathway inhibitor, 2 μM) or JM91 (0.5 μM), respectively or in combination in a serum-free medium for 24 h. The cells were then exposed to 25 ng/mL PDGF-BB for 20 h before 1 μCi/mL [3H]thymidine had been added to the medium. Four hours later, the labeling reaction was terminated and quantified using a liquid scintillation counter. The data is expressed as the mean ± S.E.M. from four different sets of experiments. *P < 0.05 and ***P < 0.005 vs only PDGF-BB-stimulated RAVSMCs.

pRb phosphorylation, which correlated well with the concentration-dependent inhibition of PDGF-BB-stimulated cell proliferation, DNA synthesis and cell cycle progression. This indicates that the inhibition of pRb phosphorylation contributes greatly to the antiproliferative activity of JM91. Considering that pRb can be phosphorylated by a number of cell cycle regulatory molecules, such as CDKs and cyclins [12,30–34], the effect of JM91 on expressions of CDK2, CDK4, cyclin E and D_1 was next examined. Accordingly, JM91 concentration-dependently inhibited CDK2, cyclin E, CDK4 and cyclin D_1 expression, indicating that cell cycle arrest in the G_1-phase might be due to the downregulation of CDKs/cyclins complex expression and pRb phosphorylation [35].

The antiproliferative mechanism of JM91 was investigated by determining if it had a direct inhibitory effect on the PDGF-BB-stimulated phosphorylation of PDGF-Rβ. As shown in Fig. 5, JM91 had no effect on the PDGF-BB-stimulated phosphorylation of PDGF-Rβ at all concentrations examined in this study. This suggests that the inhibition of JM91 on RAVSMCs proliferation does not occur at the receptor level. It was indirectly confirmed that JM91 could also inhibit the FBS-induced proliferation of RAVSMCs (Data not shown). Therefore, the effect of JM91 on the downstream signal transduction, such as ERK1/2, PI3K/Akt and PLCγ1 signaling pathways, was further investigated. ERK1/2 plays a key role in VSMCs growth regulation by PDGF-BB [36–38]. PDGF-BB activates the ERK1/2 pathway by triggering RAS-RAF activation, MEK1 phosphorylation, and ERK1/2 phosphorylation [39]. PI3K/Akt, another important signaling pathway, is triggered by PDGF-BB. It was recently reported that this pathway is involved in the anti-apoptotic effect of PDGF-BB in VSMCs [40]. Many studies suggested that the MAPK pathway and Akt pathway lead to two distinct end effectors, which are regulated independently by various stimulators and inter-

mediate signal transduction molecules [41]. However, recent studies suggest that the two pathways also cross talk, possibly through an interaction between Raf and Akt [42,43]. As shown in Figs. 6 and 7, JM91 inhibited the 25 ng/mL PDGF-BB-stimulated phosphorylation of ERK1/2 and PI3K/Akt at various time points and in a concentration-dependent manner. In this experiment, the total ERK1/2, Akt and PLCγ1 were used as a control for protein loading. However, the total amount of Akt rapidly decreased upon its activation. Mayumi et al. [44] reported that PDGF caused a rapid decrease in the Akt protein levels concomitant with Akt activation. PDGF causes the regulated proteolytic downregulation of Akt, which is dependent on PI3K and proteasome activities. The proteasome-dependent downregulation of Akt might be a fundamental mechanism to regulate the activity and function of Akt in VSMCs. In agreement with these findings, a potent synergistic inhibition of DNA synthesis can be observed when cells were treated with a low concentration of U0126 (a well-known ERK1/2 inhibitor) and LY294002 (a well-known PI3K/Akt pathway inhibitor) in combination. In addition, when JM91 were treated to cells in combination with either or both of the inhibitors, a potent additive/synergistic inhibition of DNA synthesis can be observed (Fig. 8). These results supported the hypothesis that the inhibitory effects of JM91 on ERK1/2 and Akt activation can be effective to block PDGF-BB-stimulated RAVSMCs proliferation.

In summary, this study showed that JM91 inhibited the proliferation and DNA synthesis of PDGF-BB-stimulated RAVSMCs by suppressing ERK1/2 and PI3K/Akt signaling. Furthermore, JM91 arrests cell cycle progression in the G_0/G_1 phase by downregulating the expression of the cyclin D_1, cyclin E, CDK4 and CDK2 proteins as well as phosphorylation of the Rb protein. This suggests that JM91 might be useful for preventing the progression of vascular complications, such as restenosis after percutaneous transluminal coronary angioplasty and atherosclerosis.

Acknowledgments

This study was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (The Regional Research Universities Program/Chungbuk BIT Research-Oriented University Consortium), and by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MOST: R01-2006-000-10020-0).

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