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YSK2821, a newly synthesized indoledione derivative, inhibits cell proliferation and cell cycle progression via the cell cycle-related proteins by regulating phosphatidylinositol-3 kinase cascade in vascular smooth muscle cells

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ABSTRACT

Indoledione derivatives have pronounced biological effects, i.e., cytotoxic activities against cancer cell lines and antifungal and antibacterial activities. The present study was designed to investigate the effects of YSK2821, a newly synthesized indoledione derivative, on platelet-derived growth factor (PDGF-BB)-induced vascular smooth muscle cell (VSMC) proliferation, as well as the molecular mechanisms of the anti-proliferative effects of YSK2821 in VSMCs. We found that YSK2821 caused the accumulation of cells in the G1 phase of the cell cycle and inhibited [^{3}H]-thymidine incorporation. We demonstrated that YSK2821 remarkably decreased Akt kinase phosphorylation as the mechanism by which YSK2821 suppressed cell signal transduction events in VSMC proliferation. Furthermore, in terms of the effects of YSK2821 on cell cycle-related proteins, YSK2821 enhanced the expression of the cyclin-dependent protein kinase (CDK) inhibitor p27 and down-regulated CDK2 and cyclin E expression, but did not affect CDK4 and cyclin D1 expression. YSK2821 also inhibited the phosphorylation of Rb, a key regulator in the cell cycle. These results indicate that YSK2821, a newly synthesized indoledione derivative, may inhibit VSMC proliferation via a phosphatidylinositol (PI)-3 kinase-dependent pathway, and thus shed light on a novel role for YSK2821 as a potential preventive regulator of cardiovascular disease.

1. Introduction

During progression of atherosclerotic lesions, vascular smooth muscle cell (VSMC) proliferation is of particular pathophysiologic importance (Gordon et al., 1990). In atherosclerotic lesions, VSMCs are exposed to mitogenic substances such as platelet-derived growth factor (PDGF) (Cui et al., 1998; Gutstein et al., 1999). Moreover, the association between PDGF and VSMC proliferation has been demonstrated in animal experiments, in which increases of PDGF-BB after arterial injury were correlated with neointimal cellular proliferation (Uchida et al., 1996).

PDGF-induced mitogenesis and proliferation are also known to be prerequisites for the intimal thickening that is observed after angioplasty (Sachinidis et al., 1990). PDGF-BB binding to PDGF-receptor beta chain (PDGF-Rβ) leads to phosphorylation of the receptor at multiple tyrosine residues. This activated PDGF-Rβ is associated with various signaling pathways, including those of extracellular signal-regulated kinase (ERK)1/2, Akt and phospholipase (PLC)γ1 (Claesson-Welsh, 1994; Heldin et al., 1998; Mulvany, 1990).

Many growth factors such as insulin-like growth factor (IGF)-1 and PDGF bind to their respective receptors and activate phosphatidylinositol (PI)-3 kinase. As a downstream target of PI-3 kinase, Akt can induce a variety of biological responses. Akt is amplified or over-expressed in gastric adenocarcinomas, breast cancer, hepatocarcinoma, and prostate carcinoma, and its activation correlates to cancer progression (Sekine et al., 2007). Active Akt inhibits apoptosis and stimulates cell cycle progression by phosphorylating numerous targets in various cell types, including cancer cells. Consequently, inhibitors of Akt activity may be powerful anticancer agents (Mullany et al., 2007).

The indoledione derivatives have pronounced biological effects, i.e., cytotoxic activities against cancer cell lines (Beall and Winski, 2000), as well as antifungal (Ryu et al., 2000, 2007) and antibacterial activities (Fukuyama et al., 1998). However, the anti-proliferative effect of YSK2821 on VSMCs is not understood.

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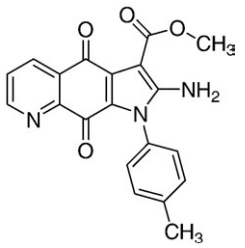

Fig. 1. Chemical structure of YSK2821, a newly synthesized indoledione derivative.

In the present study, we sought to elucidate the anti-proliferative effect and the molecular role of YSK2821 in PDGF-BB-stimulated signaling. Our findings provide evidence that YSK2821, a newly synthesized indoledione derivative, can inhibit VSMC proliferation and cell cycle progression via the cell cycle-related proteins by regulating PI-3 kinase cascade in VSMCs.

2. Materials and methods

2.1. Materials

Cell culture materials were purchased from Invitrogen (Carlsbad, CA). Anti-phospho-ERK1/2, anti-phospho-Akt, anti-phospho-PLCγ1, anti-ERK1/2, anti-Akt and anti-PLCγ1 antibodies were from Cell Signaling Technology Inc. (Beverly, MA). PDGF-BB, anti-phospho-PDGF-Rβ and anti-PDGF-Rβ polyclonal antibody were obtained from Upstate Biotechnology (Lake Placid, NY). Other chemicals were of analytical grade.

2.2. Synthesis of YSK2821

2.2.1. Experimental

All melting points were measured with the Büchi melting point B-545 apparatus and were uncorrected. ^{1}H-NMR spectra were recorded on Varian Unity INOVA 400 MHz FT-NMR spectrometer using DMSO$_{-d6}$

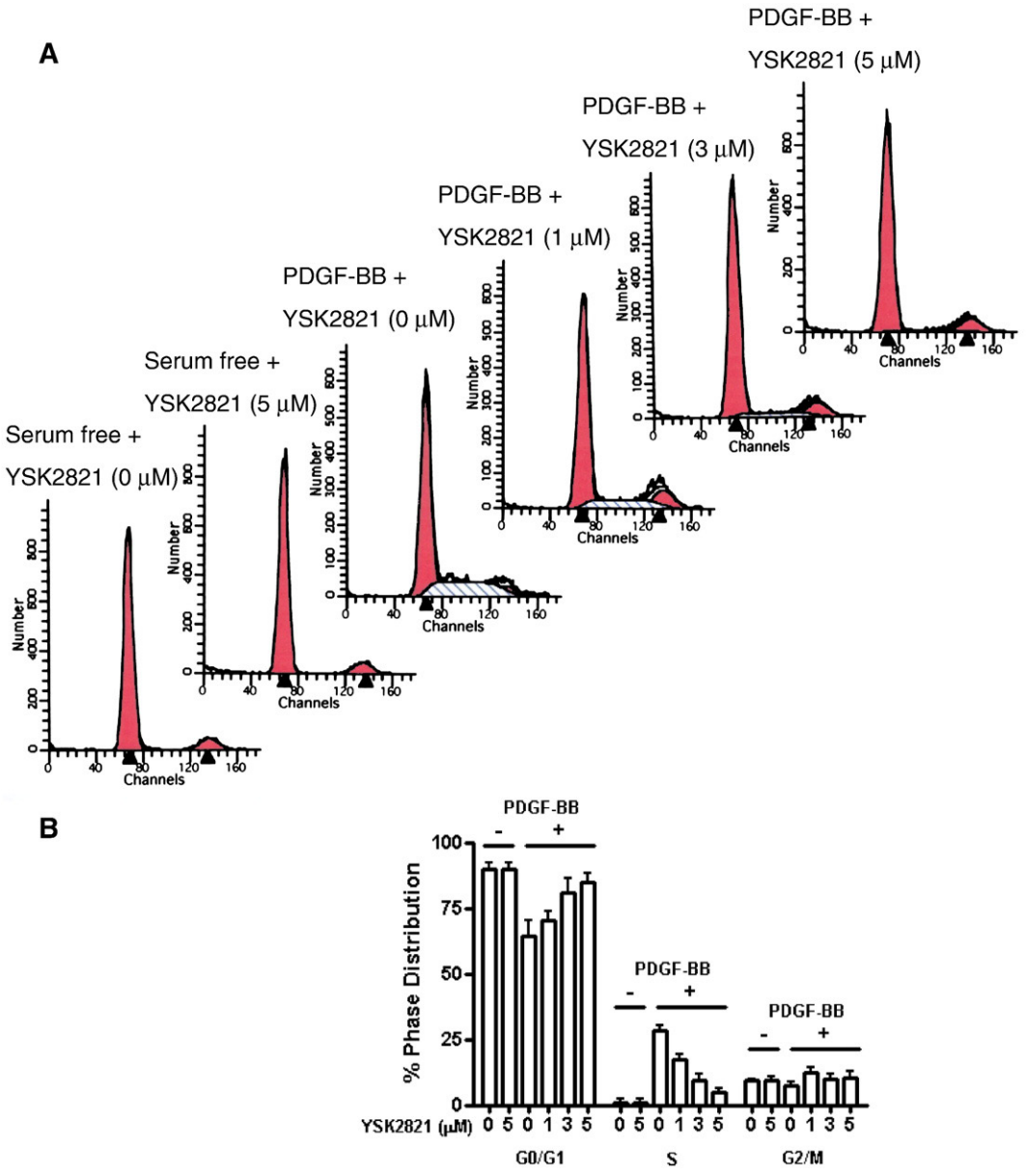

Fig. 2. Effect of YSK2821 on PDGF-BB-induced cell cycle progression. The cells were pre-cultured in the presence or absence of YSK2821 (1–5 μM) in serum-depleted medium for 24 h, and then VSMCs were stimulated with 25 ng/ml PDGF-BB. After 24 h, individual nuclear DNA content is reflected as the fluorescence intensity of incorporated propidium iodide. Each item is derived from a representative experiment, where data from at least 10,000 events were obtained (A). The cell populations in G1, S and G2/M phases were determined using the computer program, ModFitLT (B). Data are representative of at least three independent experiments with similar results.

or $CDCl_3$ with TMS. Mass spectra were taken with Jeol JMS AX505 WA. The reagents were purchased from Aldrich Chemical Co. YSK2821 (methyl 2-amino-4,9-dihydro-4,9-dioxo-1-*p*-tolyl-1H-pyrrolo[3,2-g] quinoline-3-carboxylate) was prepared by cyclizing 6,7-dichloroquinoline-5,8-dione with methyl cyanoacetate and arylamine according to the known method (Ryu et al., 2007).

2.2.2. YSK2821

mp 262–263 °C; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 2.37 (s, 3H, methyl), 3.92 (s, 3H, methoxy), 6.98 (d, 1H, benzene), 7.14 (d, 1H, benzene), 7.50 (m, 1H, benzene), 7.66 (q, 1H, 2-pyridine), 7.91 (m, 1H, benzene), 8.43 (q, 1H, 2-pyridine), 9.09 (q, 1H, 2-pyridine); MS (m/z) 361 (M^+). YSK2821 was dissolved in dimethylsulfoxide (DMSO) and added to Dulbecco's modified Eagle's medium (DMEM) with a maximum final DMSO concentration of 0.05% (Fig. 1).

2.3. Vascular smooth muscle cell culture

VSMCs were isolated by enzymatic dispersion as previously described (Kim et al., 2002). VSMCs were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 8 mM HEPES, 2 mM L-glutamine at 37 °C in a humidified 95% air and 5% CO_2 incubator. The purity of VSMCs in culture was confirmed by Western blotting of α-smooth muscle actin. VSMCs were used at passages 4–8.

2.4. Cell count and [^{3}H]-thymidine incorporation assay

VSMCs were seeded into 12-well culture plates at 1×10^4 cells/ml and then cultured in DMEM containing 10% FBS at 37 °C for 24 h until 70% confluent. The media were then replaced by serum-free media containing YSK2821. Cells were stimulated with 25 ng/ml PDGF-BB for 24 h, trypsinized with trypsin-EDTA, and counted using a hemocytometer. For [^{3}H]-thymidine incorporation experiments, VSMCs were seeded on 24-well culture plates at 5000 cells/well and then allowed to grow for 3–4 days in DMEM. They were then placed in serum-free media with or without YSK2821 (1–5 μM) for 20 h and exposed to media with 25 ng/ml PDGF-BB. VSMCs were pulsed with 1 μCi/well [^{3}H]-thymidine for 4 h. The labeling reaction was terminated by aspirating the medium and subjecting cultures to sequential washes on ice with 1×PBS containing 10% trichloroacetic acid and ethanol/ether (1:1 v/v). Acid-insoluble [^{3}H]-thymidine was extracted into 250 μl of 0.5 M NaOH per well, and 100 μl of the extract was mixed with a liquid cocktail (Ultimagold, Packard Bioscience, Shelton, CT) and counted in a scintillation counter (model LS3801, Beckman, Germany).

2.5. Cell cycle analysis

VSMCs in 60-mm^2 dishes were incubated in DMEM without serum (Invitrogen) with or without YSK2821 (1–5 μM) for 24 h. The VSMCs were then treated with or without 25 ng/ml PDGF-BB for 24 h, and then trypsinized and centrifuged at 1500 ×*g* for 7 min. Centrifuged pellets were suspended in 1 ml of 1×PBS, washed twice, and re-centrifuged. Pellets were suspended in 70% ethanol and fixed overnight at 4 °C. The fixed VSMCs were briefly vortexed and centrifuged at 15,000 ×*g* for 5 min. Ethanol was discarded and pellets were stained with 0.4 ml of propidium iodide (PI) solution (50 μg/ml PI in buffer containing 100 μg/ml of RNase A). Samples were incubated for 1 h at room temperature before analysis by flow cytometry. The PI-DNA complex in each cell nucleus was measured using a FACSCalibur (BD Biosciences, San Jose, CA). The rates of G0/G1, S and G2/M phases were determined using the computer program ModFitLT (Verity Software House, Topsham, ME).

2.6. Cell death analysis

Cell death including apoptosis was assessed by staining with Alexa Fluor 488-labeled annexin V (Annexin V-FLUOS Staining Kit, Roche, Germany). After the incubations, both floating and adherent cells were pooled and centrifuged for 5 min at 1000 ×*g*. Pelleted cells were washed in cold phosphate-buffered saline (PBS). Thereafter, cells were centrifuged again for 5 min at 1000 ×*g* and resuspended in 100 μl Annexin-Binding Buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$ pH 7.4) yielding a cell density of 1×10^6 cells/ml. Five microliters of annexin V conjugate and 1 μl of 100 μg/ml PI working reagent were added to each 100 μl of cell suspension. The cells were incubated at room temperature for 15 min. After the incubation period, 400 μl of Annexin-Binding Buffer was added with gentle mixing, and the samples were kept on ice. The stained cells were analyzed by flow cytometry, where the fluorescence emission was measured at 530 nm (Alexa Fluor 488). The rate of cell death was determined using the computer program ModFitLT (Verity Software House, Topsham, ME).

2.7. Western blot analysis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on VSMC lysates using 10% acrylamide gels, as described by Laemmli (1970). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK). Membranes were blocked overnight at 4 °C in Tris-buffered saline containing 0.1% Tween 20 (TBS/T) and 5% skim milk powder, and then incubated with a 1:2000 dilution of anti-phospho-ERK1/2, anti-phospho-Akt, anti-phospho-PLCγ1, anti-ERK1/2, anti-Akt, anti-PLCγ1 anti-phospho-PDGF-Rβ or anti-PDGF-Rβ antibodies. Blots were then washed with TBS/T and incubated with a 1:5000 dilution of horseradish

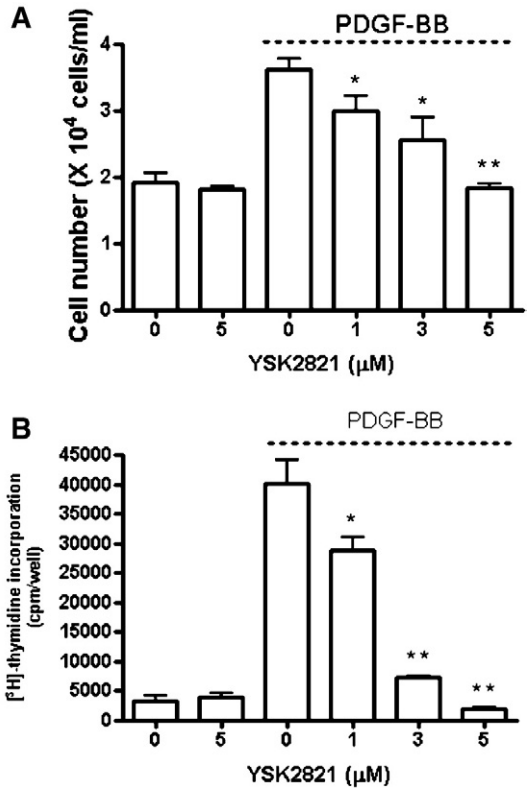

Fig. 3. Effect of YSK2821 on PDGF-BB-induced cell proliferation. The cells were pre-cultured in serum-free medium in the presence or absence of YSK2821 (1–5 μM) for 24 h, and then stimulated with 25 ng/ml PDGF-BB for 24 h. Cells were trypsinized and then counted with a hemocytometer (A). The cells were pre-cultured in serum-free medium in the presence or absence of YSK2821 (1–5 μM) for 24 h, and then stimulated with 25 ng/ml PDGF-BB for 20 h. [^{3}H]-thymidine (1 μCi/ml) was added to the medium and the cells were incubated for an additional 4 h. The incorporated radioactivity was determined using a liquid scintillation counter (B). Each value represents mean±S.D. from three replicates. $^*P<0.05$ and $^{**}P<0.01$ indicate statistically significant differences versus the PDGF-BB-stimulated group.

Table 1
Effect of YSK2821 on apoptosis or necrosis of PDGF-BB-stimulated VSMCs

	PDGF-BB(−)		PDGF-BB(+)			
YSK2821 (μM)	0	5	0	1	3	5
Annexin V^-/PI^+	0.05±0.01	0.06±0.04	0.02±0.01	0.15±0.12	0.08±0.02	0.08±0.02
Annexin V^+/PI^+	3.15±0.55	4.66±0.09	3.72±0.18	4.81±2.67	4.12±1.20	5.20±0.55
Annexin V^-/PI^-	92.46±0.49	89.95±3.93	93.60±0.27	91.63±4.60	92.96±2.79	89.40±5.08
Annexin V^+/PI^-	4.35±0.05	5.38±3.99	2.66±0.09	3.41±1.81	2.85±1.58	5.32±4.51

The cells were pre-cultured in serum-free medium in the presence or absence of YSK2821 (1–5 μM) for 24 h, and then stimulated with 25 ng/ml PDGF-BB for 24 h. Apoptotic (Annexin V^+/PI^-) or necrotic cells (Annexin V^+/PI^+) were identified by using Annexin V binding and PI dye detected by flow cytometry.

peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG antibodies (New England Biolabs, Beverly, MA). The proteins were detected using an enhanced chemiluminescence (ECL) Western blotting detection reagent (ECL plus kit, Amersham Pharmacia Biotech). The phospho-ERK1/2, phospho-Akt, phospho-PLCγ1 or phospho-PDGF-Rβ was normalized to the total ERK1/2, Akt, PLCγ1, PDGF-Rβ or α-actin values, respectively. The intensities of the bands were quantified using the program, Scion-Image for Windows (Scion Corporation, Frederick, MD).

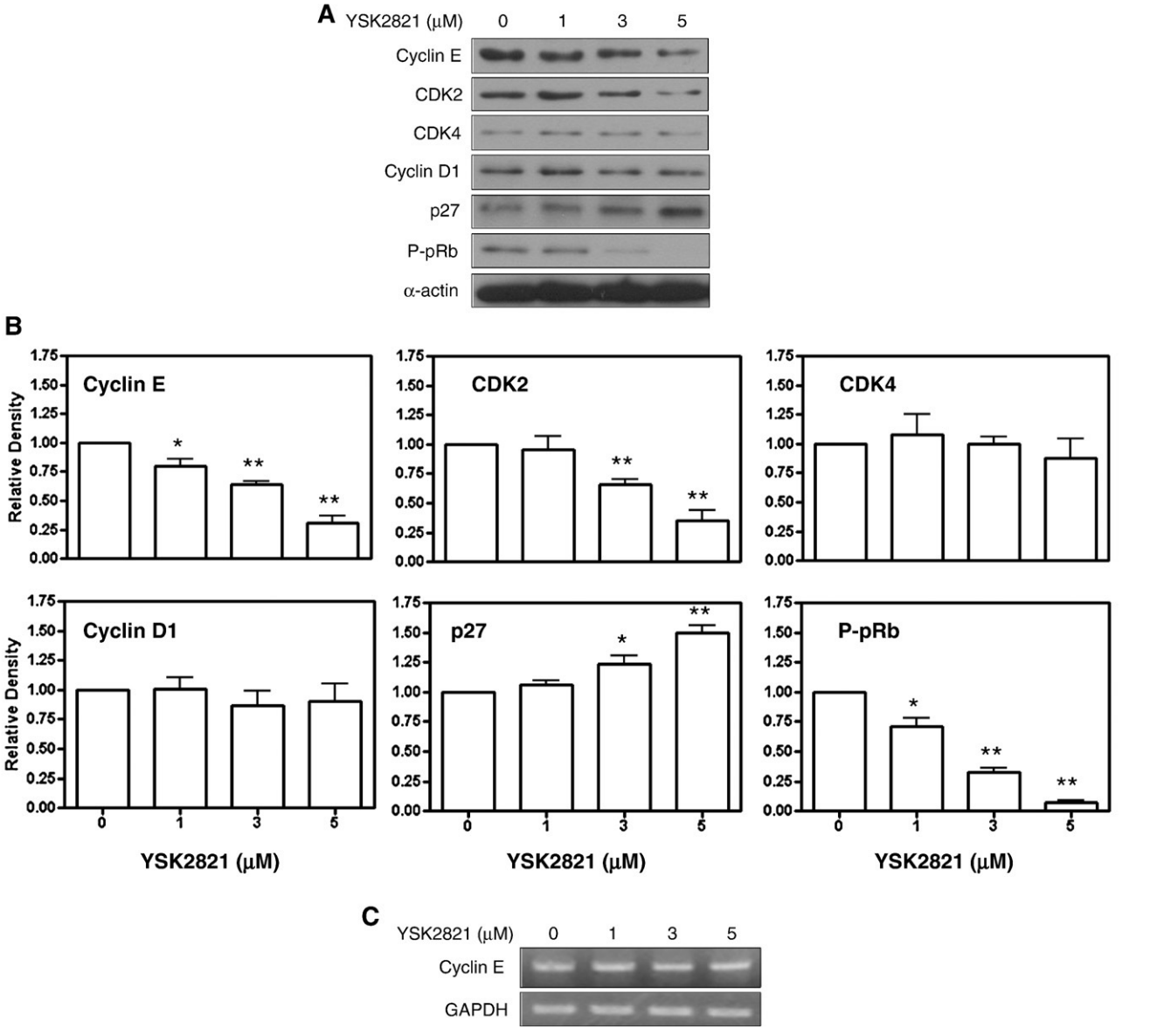

Fig. 4. Effect of YSK2821 on PDGF-BB-induced cell cycle-related proteins in VSMCs. The cells were cultured in 12-well plates until confluent, and the medium was then replaced with serum-free medium in the presence or absence of YSK2821 (1–5 μM) for 24 h. VSMCs were then stimulated with 25 ng/ml PDGF-BB for 24 h. The cells were lysed, and proteins from cell lysates were resolved by SDS-PAGE, transferred to a PVDF membrane and blotted with anti-CDK2, anti-cyclin E, anti-CDK4, anti-cyclin D1, anti-p27, anti-phospho-Rb or anti-α-actin antibodies to analyze protein expression. Similar results were obtained in three independent experiments (A). Bands were quantified by densitometric analysis, and the results are shown as relative density compared with control (B). Confluent cells were pre-treated with YSK2821 in serum-free medium for 24 h, and then stimulated with PDGF-BB for 24 h. The RT-PCR was performed as described in Materials and methods. The GAPDH gene was used for normalization. Similar results were obtained in three independent experiments. (C). $^*P<0.05$, $^{**}P<0.01$ *vs.* PDGF-BB alone.

2.8. RNA isolation and semi-quantitative RT-PCR

RNA extraction and analysis of VSMCs were performed as previously described (Kim and Yun, 2007). In brief, total RNA was isolated using the RNeasy Mini Kit according to the manufacturer's protocol (Qiagen GmbH, Germany). RNA concentrations were determined by measuring absorbance at 260 nm. Total RNA (1 μg) was subjected to RT-PCR (Bioneer, Korea) to co-amplify a cyclin E fragment and the GAPDH gene (Kim and Yun, 2007). The sequences of the oligonucleotide primers used for RT-PCR were 5′-ACATTCTACTTGGCACAGG-3′ (sense) and 5′-TGTCTCCTGCTCACTGCT-3′ (antisense) for cyclin E, and 5′-CCTGCACCACCAACTGCTTA-3′ (sense) and 5′-GATGCCAGTGAGCTTCCCGT-3′ (antisense) for GAPDH. After an initial denaturation step at 94 °C for 5 min, the PCR (Eppendorf, Hamburg, Germany) reaction was continued for an additional 30 cycles. The cycle profile for cyclin E and GAPDH gene amplification was 94 °C, 30 s; 52 °C, 30 s and 72 °C, 1 min. A final extension was performed at 72 °C for 5 min.

2.9. Akt kinase activity assay

A sensitive and quantitative *in vitro* AKT kinase assay, based on an AKT kinase kit from Cell Signaling (Beverly, MA), was established to screen YSK2821, and Akt kinase activity was measured in a colorimetric ELISA assay using the following reaction conditions: 25 mM Tris–HCl, pH 7.5, 5 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$, 2 mM DTT, 200 μM ATP, 1.5 μM biotinylated Akt-substrate peptide, and 10 ng recombinant Akt. The absorbance was measured at 450 nm with a microtiter plate reader (Bio-Rad, Japan).

2.10. Statistical analysis

Experimental results are expressed as means ± S.D. One-way analysis of variance (ANOVA) was used for multiple comparisons, and this was followed by Dunnett's test. Differences at the $^{*}P<0.05$ and $^{**}P<0.01$ levels were considered statistically significant.

3. Results

3.1. Effect of YSK2821 on PDGF-BB-induced cell cycle progression

The proliferation of VSMCs in arterial walls is involved in cardiovascular problems, including atherosclerosis, and one of the principal regulators of VSMC proliferation is PDGF-BB (Ross, 1993; Heldin et al., 1998). Therefore, inhibition of abnormal proliferation of PDGF-BB-induced VSMCs may be an effective cardiovascular therapy (Myllarniemi et al., 1999). To determine the effects of YSK2821 on the cell cycle progression of VSMCs, flow cytometry was performed using propidium iodide. As shown in Fig. 2, YSK2821 induced accumulation of cells in the G1 phase of the cell cycle in a concentration-dependent manner.

3.2. Effect of YSK2821 on PDGF-BB-induced cell proliferation

We assessed the inhibitory effects of YSK2821 by direct cell counting. VSMCs were pre-cultured in the presence of YSK2821 (1–5 μM) in serum-depleted medium for 24 h and then stimulated with 25 ng/ml PDGF-BB for 24 h. Pre-treatment with YSK2821 suppressed the PDGF-BB-stimulated cell numbers in a concentration-dependent manner. The inhibition percentages were 33.4 ± 9.1, 61.3 ± 16.3 and 97.5 ± 4.3% at 1, 3 and 5 μM YSK2821, respectively (Fig. 3A). The antiproliferative activity of YSK2821 was also assessed by [^{3}H]-thymidine incorporation assays. As shown in Fig. 3B, YSK2821 reduced PDGF-BB-induced [^{3}H]-thymidine incorporation in a concentration-dependent manner. Significant inhibition of [^{3}H]-thymidine incorporation after PDGF-BB stimulation was observed at 1 μM (31%, $P<0.05$), 3 μM (89%, $P<0.01$) and 5 μM (99%, $P<0.01$) YSK2821. Therefore, our results indicate that YSK2821 induces arrest of cell cycle progression and inhibits of VSMC proliferation.

3.3. Effect of YSK2821 on apoptosis

The apoptotic effects of YSK2821 were measured in VSMCs under the same conditions. Apoptotic (annexin V^+/PI^-) or necrotic cells (annexin V^+/PI^+) were identified by means of annexin V binding and PI dye detected by flow cytometry. As shown in Table 1, at the concentrations used in this study, YSK2821 did not significantly increase apoptosis.

3.4. Effect of YSK2821 on PDGF-BB-induced cell cycle-related proteins

Since cell division and growth are tightly controlled by a series of positive and negative regulators in the cell cycle of mammalian cells, we analyzed the effects of YSK2821 on PDGF-BB-induced cell cycle-related proteins. As shown in Fig. 4, YSK2821 enhanced the expression of the CDK inhibitor, p27. YSK2821 also decreased expression of CDK2 and cyclin E, but not of CDK4 and cyclin D1. Furthermore, YSK2821 inhibited the phosphorylation of Rb, a key regulator of the G1 to S phase transition in the cell cycle. To confirm whether down-regulation of cyclin E was due to its degradation or inhibition of cyclin E mRNA production, RT-PCR was performed. At the concentrations used in this study, YSK2821 did not significantly change cyclin E mRNA levels. Therefore, our results suggest that down-regulation of Rb protein and

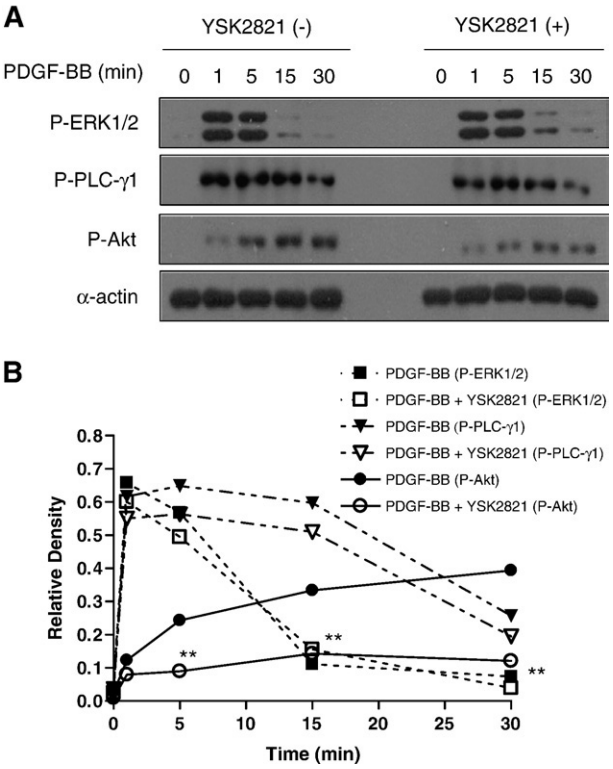

Fig. 5. Time course of YSK2821 effects on PDGF-BB-induced phosphorylation of PLCγ1, ERK1/2 and Akt. The cells were cultured in 6-well plates until confluent, and the medium was then replaced with serum-free medium in the presence or absence of YSK2821 (5 μM) for 24 h. The cells were then stimulated with 25 ng/ml PDGF-BB for 0–30 min. Equal amounts of total proteins (40 μg) were subjected to 10% SDS-PAGE, and phosphorylation of PLCγ1, ERK1/2 and Akt proteins was detected by Western blotting using specific antibodies. α-actin protein was used as an internal control (A). Quantification of band intensities from three independent experiments was determined by densitometry. Data are reported as mean ± S.D. from three experiments performed in triplicate for P-PLCγ1, P-ERK1/2 or P-Akt/α-actin (B). $^{**}P<0.01$ indicates statistically significant differences versus the PDGF-BB-induced group.

up-regulation of the CDK inhibitor, p27 protein, but not of mRNA expression, contribute to the anti-proliferative effects of YSK2821 on PDGF-BB-induced VSMCs.

3.5. Effect of YSK2821 on PDGF-BB-induced ERK1/2, PLCγ1, Akt and PDGF-receptor β chain phosphorylation

Much evidence indicates that ERK1/2, Akt and PLCγ1 are activated by PDGF-BB stimulation (Kim et al., 2005). Therefore, the inhibitory effects of YSK2821 on ERK1/2, PLCγ1 and Akt levels as possible PDGF-Rβ downstream signaling pathways were examined by Western blotting. Upon PDGF-BB stimulation for 1–30 min, Akt activation was dramatically increased in VSMCs, and co-treatment of cells with PDGF-BB and 5 μM YSK2821 significantly inhibited Akt activation in a time-dependent manner (Fig. 5). However, activation of ERK1/2 and PLCγ1 was not affected by YSK2821 in PDGF-BB-induced VSMCs. In addition, the levels of phosphorylated Akt stimulated by PDGF-BB were significantly inhibited by YSK2821 in a concentration-dependent manner (Fig. 6). Total ERK1/2, PLCγ1, PDGF-Rβ, and α-actin levels were not altered by YSK2821. However, the total Akt level was decreased by addition of PDGF-BB. Densitometric analysis showed that the relative intensity of phosphorylated Akt kinases/α-actins in the presence of 1, 3 and 5 μM YSK2821 was 66%, 35% and 20% as compared with PDGF-BB alone. We also addressed whether YSK2821 influenced PDGF-BB-induced activation of PDGF-Rβ tyrosine kinase. Increased levels of phosphorylated PDGF-Rβ tyrosine kinase were detected after stimulation of VSMCs with PDGF-BB (25 ng/ml) for 5 min compared with those in untreated cells. Phosphorylation of PDGF-Rβ tyrosine kinase by PDGF-BB was not affected by pretreatment with YSK2821 (Fig. 6). In order to demonstrate whether YSK2821 inhibits VSMC proliferation by regulating direct Akt kinase activation, we also tested the effects of YSK2821 on Akt kinase activation using an Akt-GST fusion protein.

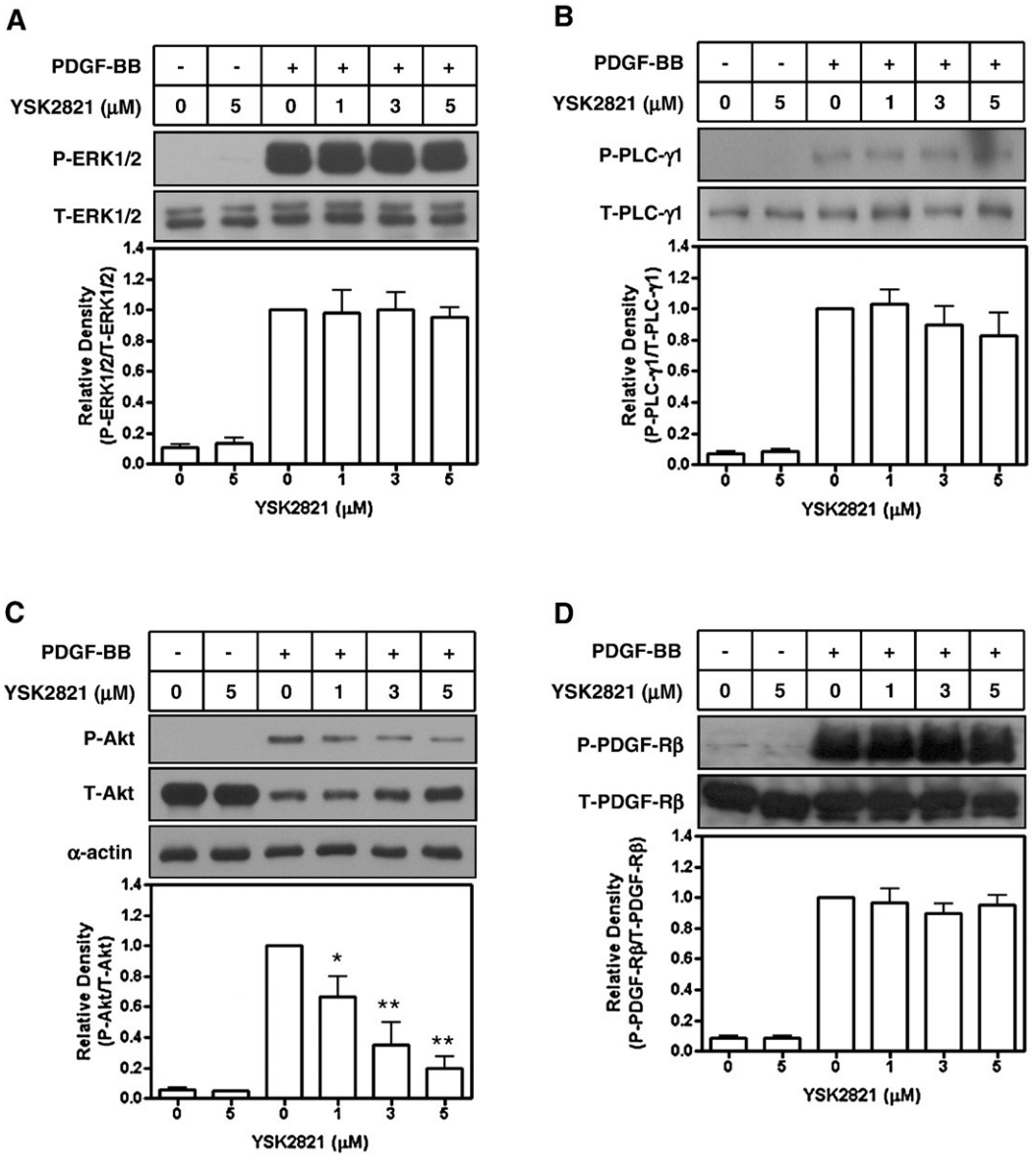

Fig. 6. Effect of YSK2821 on PDGF-BB-induced phosphorylation of PLCγ1, ERK1/2, Akt and PDGF-Rβ. The cells were cultured in 6-well plates until confluent. The medium was then replaced with serum-free medium in the presence or absence of YSK2821 (1–5 μM) for 24 h, and the cells were then stimulated with 25 ng/ml PDGF-BB for 5–20 min. Equal amounts of total proteins (40 μg) were subjected to 10% SDS-PAGE, and phosphorylation of PLCγ1, ERK1/2, Akt and PDGF-Rβ protein was detected by Western blotting using specific antibodies. Total PLCγ1, ERK1/2, Akt and PDGF-Rβ proteins were used as internal controls. Quantification of band intensities from three independent experiments was determined by densitometry. Data were described as mean ± S.D. from three experiments performed in triplicate for P-PLCγ1/T-PLCγ1, P-ERK1/2/T-ERK1/2, P-Akt/T-Akt or P-PDGF-Rβ/T-PDGF-Rβ. $^{*}P<0.05$ and $^{**}P<0.01$ indicate statistically significant differences versus the PDGF-BB-induced group.

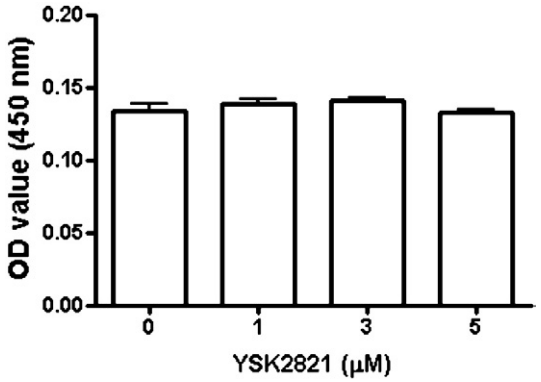

Fig. 7. Effect of YSK2821 on Akt kinase activity. Recombinant Akt kinase was used to measure *in vitro* kinase activity treated with YSK2821 at 0, 1, 3, 5 μM as described in Materials and methods. Data were described as mean±S.D. from three experiments performed in triplicate.

However, the Akt kinase activities were not significantly inhibited by YSK2821 (Fig. 7). These results indicate that, among possible intracellular signaling pathways, inhibition of PI-3 kinase cascade, but not direct inhibition of Akt activation, may be involved in the YSK2821-induced inhibition of cell cycle progression and VSMC proliferation.

4. Discussion

Cardiovascular diseases are associated with a multitude of pathophysiologic conditions, such as inflammation, pulmonary hypertension, coronary artery restenosis following balloon angioplasty, and VSMC proliferation in response to vessel injury (Ross, 1993). Therefore, inhibition of VSMC proliferation is important in the treatment of cardiovascular disease. In the present study, we investigated the anti-proliferative activity of YSK2821 on VSMCs and the related signal transduction in cultured VSMCs. The experimental results showed that YSK2821 dose-dependently inhibited the increase in [^{3}H]-thymidine incorporation in response to stimulation with 25 ng/ml PDGF-BB (Fig. 3). Furthermore, PDGF-BB-stimulated cell cycle progression was also potently inhibited by YSK2821 (Fig. 2). These results indicate that YSK2821 has a potent inhibitory effect on PDGF-BB-stimulated responses in VSMCs.

In the cell cycle of mammalian cells, cell division and growth are tightly controlled by a series of positive and negative regulators that act at sequential points throughout the cell cycle. We found that the inhibitory effect of YSK2821 on VSMCs is exerted at some point in the G1 phase. Therefore, we specifically focused on proteins that regulate the cell cycle. CDKs play a central role and promote G1/S transition by phosphorylation of the Rb protein (Weinberg, 1995; Sriram and Patterson 2001). Intrinsic CDK inhibitors, such as p27, bind to the CDK-cyclin complex and inhibit its kinase activity (Peter and Herskowitz, 1994). p27 induces G1 arrest via suppression of CDK2 activity by binding to the cyclin E-CDK2 complexes (Peter and Herskowitz, 1994). We found that YSK2821 enhanced the expression of the CDK inhibitor p27. YSK2821 also down-regulated CDK2 and cyclin E expression, but not CDK4 and cyclin D1 expression. Furthermore, YSK2821 inhibited the phosphorylation of Rb, a key regulator of the G1 to S phase transition in the cell cycle (Fig. 4). Our results suggest that down-regulation of Rb protein and up-regulation of the CDK inhibitor, p27, contribute to the anti-proliferative effect of YSK2821 on VSMCs.

Here, the effects of YSK2821 on these intracellular signaling cascades were examined. The up-regulation of PDGF-R expression is associated with the development and progression of cardiovascular diseases such as hypertension (Mulvany, 1990) and atherosclerosis (Ross, 1993; Majesky et al., 1990). Induction of cell survival or proliferation is a highly regulated process and can be promoted by PDGF-BB (Kim and Yun, 2007; Sachinidis et al., 1990). In our study, PDGF-BB-induced PDGF-Rβ tyrosine phosphorylation in VSMCs is not altered by YSK2821 (Fig. 6). A number of receptor tyrosine kinases, including the receptors for PDGF-BB, activate PI3-kinase/Akt, ERK1/2 kinase and PLCγ1, and their signaling pathways are important in early intracellular mitogenic signal transduction for cell growth and survival (Muller, 1997). Among the MAP kinase family, ERK1/2 has been implicated in the growth of various cell types (Hommes et al., 2003; Robinson and Cobb, 1997). PI3-kinase positively regulates cell survival. Akt, a serine/threonine protein kinase, is activated through the PI3-kinase pathway (Higaki and Shimokado, 1999). PLCγ1 signaling mediates a central downstream signal transduction route for various growth factors, including PDGF-BB (Heldin et al., 1998). These kinases have each been implicated in VSMC proliferation, cell cycle progression, and cell survival (Higaki and Shimokado, 1999; Waltenberger, 1997). The current study shows that YSK2821 is able to suppress the enhanced growth in the presence of PDGF-BB *in vitro*. Levels of PLCγ1, ERK1/2, and Akt kinase phosphorylation are increased by PDGF-BB, and YSK2821 inhibited PDGF-BB-induced phosphorylation of Akt, but not of PLCγ1 or ERK1/2, indicating that Akt protein is a potential target for YSK2821 (Fig. 5). Akt, referred to as PKB or Rac, plays a critical role in controlling survival and apoptosis (Franke et al., 1997, 1995; Burgering and Coffer, 1995). This protein kinase is activated by insulin and various growth and survival factors in a wortmannin-sensitive pathway involving PI-3 kinase (Burgering and Coffer, 1995; Franke et al., 1995). In addition to its role in survival, PI-3 kinase/Akt is involved in cell cycle regulation by negatively regulating the cyclin-dependent kinase inhibitor, p27 Kip (Gesbert et al., 2000). In our result, YSK2821 did not inhibit VSMC proliferation by regulating direct Akt kinase activation (Fig. 7). At the present stage, the molecular mechanism by which the YSK2821 exerts it influence on VSMC proliferation has not been fully explained. However, our results indicate that, inhibition of PI-3 kinase/Akt cascade induced by PDGF-BB may be involved in the YSK2821-induced inhibition of cell proliferation. Compounds for inhibition of PI-3 kinase pathway have been used widely in treatment of hypertension and angina, and exhibit an array of biological effects in the cardiovascular system. In animal models and in patients, these inhibitors prevent or reduce the pathology in atherosclerosis and hypertensive remodeling of resistance arteries (Myllarniemi et al., 1999).

In conclusion, our study demonstrated that YSK2821 inhibits VSMC proliferation and cell cycle progression via the cell cycle-related proteins by regulating PI-3kinase/ Akt cascade in VSMCs.

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