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The Inhibitory Effect and Mechanism of Luteolin 7-Glucoside on Rat Aortic Vascular Smooth Muscle Cell Proliferation

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The abnormal proliferation of aortic vascular smooth muscle cells (VSMCs) plays a central role in the pathogenesis of atherosclerosis and restenosis after angioplasty and possibly also in the development of hypertension. The present study was designed to examine the inhibitory effects and the mechanism of luteolin 7-glucoside (L7G) on the platelet-derived growth factor (PDGF)-BB-induced proliferation of VSMCs. L7G significantly inhibited the PDGF-BB-induced proliferation and the DNA synthesis of the VSMCs in a concentration-dependent manner. Pre-incubation of the VSMCs with L7G significantly inhibited the PDGF-BB-induced extracellular signal-regulated kinase 1/2 (ERK1/2), Akt and the phospholipase C (PLC)-γ1 activation. However, L7G had almost no affect on the phosphorylation of PDGF-β receptor tyrosine kinase, which was induced by PDGF-BB. These results suggest that L7G inhibits the PDGF-BB-induced proliferation of VSMCs *via* the blocking of PLC-γ1, Akt, and ERK1/2 phosphorylation.

Key words: Luteolin 7-glucoside (L7G), Vascular smooth muscle cell (VSMC), Platelet-derived growth factor (PDGF), Cardiovascular diseases

INTRODUCTION

Flavonoids are naturally occurring polyphenolic compounds that are present in a variety of fruits, vegetables and seeds. Flavonoids have many biological and pharmacological activities that include antioxidative, antiinflammatory, and antitumor effects (Chen *et al.*, 1990). Luteolin is a polyphenolic compound found in foods of a plant origin, and it belongs to the flavone subclass of flavonoids, which usually occur as glycosylated forms in celery, green pepper, perilla leaf and camomile tea. They have been reported to display antimutagenic, antiplatelet aggregation and anticancer effects (Shimoi *et al.*, 1998, 2000; Lu *et al.*, 2001; Casagrande and Darbon, 2001). However, the effect of L7G on VSMCs is not yet clearly understood.

Vascular smooth muscle cells are the main component of the arterial wall, and the abnormal proliferation of VSMCs plays a central role in the pathogenesis of atherosclerosis and restenosis after angioplasty, and also possibly in the development of hypertension (Ross, 1990). Since platelet-derived growth factor (PDGF) is one of the principal regulators of mitogenesis in VSMCs and the expression of PDGF is increased in atherosclerotic lesions, PDGF-induced mitogenesis and proliferation are known to be prerequisites for the intimal thickening that's seen after angioplasty (Sachinidis *et al.*, 1990). Therefore, the inhibition of VSMCs proliferation is one of the key pharmacological strategies for the prevention of cardiovascular disease.

PDGF's binding to the PDGF-Rβ leads to its phosphorylation on multiple tyrosine residues. This activated PDGF-Rβ is associated with a number of SH2 domain-containing proteins, including the p85 regulatory subunit of PI3-kinase and phospholipase C (PLC)-γ1. Of the molecules that are downstream from the PDGF-Rβ, the involvement of Shc, Grb2, PLC-γ1, and PI3-kinase in PDGF-Rβ-induced mitogenesis has been relatively well characterized. The PLC-γ1 and PI3-kinase pathways have also been shown to be important for the PDGF-induced cell cycle progression in VSMCs (Claesson-Welsh, 1994). PDGF-BB activates the extracellular regulated kinases 1 and 2 (the ERK1/2 pathway) by triggering phosphorylation

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and activation of Raf-1, (the MEK, MAPK, and ERK kinases), and ultimately the ERK1/2 kinases themselves. Activation of the ERK1/2 mitogen-activated protein kinases (MAPK) is required for mitogenic signaling through a number of tyrosine kinase growth factor receptors, and up-regulation of the PDGF receptor expression is associated with the development and progression of proliferative cardiovascular diseases, including hypertension (Mulvany, 1990) and atherosclerosis (Ross, 1995; Majesky *et al.*, 1990).

Therefore, the present study was designed to examine the effect of L7G on PDGF-BB-induced VSMC proliferation. We also investigated the inhibitory signaling pathways that are responsible for the action of L7G in VSMC proliferation.

MATERIALS AND METHODS

Materials

Luteolin 7-glucose (Sigma Chemical Co., MO, U.S.A.) was dissolved in DMSO and then further diluted in DMEM without FBS. The cell culture materials were purchased from Gibco-BRL (MD, U.S.A.). Tyrophostin AG1295 was obtained from ALEXIS Biochemicals (CA, U.S.A.). U0126, LY294002 and U73122 were obtained from Tocris (BS, UK). The phospho-ERK1/2 antibody, the phospho-Akt and phospho-PLC-γ1 antibodies were purchased from New England Biolabs (MA, U.S.A.). PDGF-BB and phospho-PDGF-R-beta chain polyclonal antibody were obtained from Upstate Biotechnology (NY, U.S.A.). The other chemicals used in the experiments were of the highest analytical grade.

Isolation and culture of rat aortic VSMCs

Rat aortic VSMCs (RASMCs) were isolated by enzymatic dispersion, as was previously described (Kim *et al.*, 2002) according to the modified method of Chamley *et al.* (Chamley *et al.*, 1977). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) that was supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 8 mM HEPES and 2 mM L-glutamine at 37°C in an incubator with a humidified atmosphere of 95% air and 5% CO_2. The purity of the VSMCs culture was confirmed by immunocytochemical localization of α-smooth muscle actin. The passage of the VSMCs used in this experiment was between 4 and 8.

Measurement of cell proliferation

For counting the cells, the VSMCs were seeded onto 12-well culture plates at 1×10^5 cells/mL, and then they were cultured in DMEM with 10% FBS at 37°C for 24 h. Under these conditions, a cell confluence of ~70% was reached. The medium was then replaced by serum-free medium containing L7G. The cells were stimulated by 50 ng/ml PDGF-BB, and then they were trypsinized by trypsin-EDTA and counted by using a hemocytometer under microscopy.

[^{3}H]-thymidine incorporation assay

For the [^{3}H]-thymidine incorporation experiments, the VSMCs were seeded onto 24-well culture plates under the same conditions as stated above, and 2 μCi/mL of [^{3}H]-thymidine was added to the medium. The reactions were terminated by aspirating the medium and subjecting the cultures to sequential washes on ice with PBS that contained 10% trichloroacetic acid and ethanol/ether (1:1, v/v). The acid-insoluble [^{3}H]-thymidine was extracted into 250 μL of 0.5 M NaOH/well, and this solution was mixed with 3 mL of scintillation cocktail (Ultimagold, Packard Bioscience, CT, U.S.A.); it was quantified by using a liquid scintillation counter (LS3801, Beckman, Düsseldorf, Germany).

Western blot analysis

The Western blotting experiment was performed as previously described (Kim *et al.*, 2002). Briefly, VSMCs in 12-well plates were incubated in DMEM medium. Rat aortic VSMCs were pre-treated with L7G for 24 h before the addition of 50 ng/mL PDGF-BB for 5 min for the ERK 1/2 and PLC-γ1 phosphorylation assay, and for 15 min for the Akt phosphorylation assay. The VSMCs were next lysed with SDS lysis buffer that contained 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM dithiothreitol (DTT) and a protease inhibitor cocktail tablet (Roche, Mannheim, Germany). The lysates were centrifuged at 13,000 × *g* for 10 min, and the supernatants were then collected. Determination of the protein of the supernatant was performed by using BCA Protein Assay Reagent (Pierce, Rockford, IL, U.S.A.) according to the manufacturer's manual with using bovine serum albumin (BSA) as a standard. The proteins were separated in 7.5–10% SDS polyacrylamide gel (PAGE) by using a Mini-Protein II System (Bio-Rad, CA, U.S.A.). The proteins were next transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) at 250 mA with a transfer buffer that contained 25 mM Tris–HCl, 192 mM glysine and 20% methanol (pH 8.3). The PVDF membrane with the proteins was blocked with 5% BSA in TBS-T at room temperature for 1 h. The membrane was washed using TBS-T and then it was incubated with primary phospho-ERK1/2, phospho-Akt, phospho-PLC-γ1 and phospho-PDGF-Rβ antibodies at 1:1000 dilutions in BSA/TBS-T buffer at 4°C for overnight, and a horseradish peroxidase-conjugated IgG secondary antibody (New England Biolabs, MA, U.S.A.) at 1:5000 dilution in BSA/TBS-T buffer was also included for 3 h at 4

°C. After washing, the proteins on the membrane were detected by a chemiluminescent reaction (ECL plus kit, Amersham Pharmacia Biotech, Buckinghamshire, UK), this was followed by exposing the membranes to Hyperfilm ECL (Amersham Pharmacia Biotech). The phospho-PDGF-Rβ, phospho-ERK1/2, phospho-Akt or phospho-PLC-γ1 was normalized by the total α-actin values, respectively. The intensities of the bands were quantified using a Scion-Image for the Windows program.

Statistical analysis

The experimental results were expressed as means ± S.E.Ms. A one-way analysis of variance (ANOVA) was used for multiple comparisons; this was followed by Dunnett's test. Differences with P values < 0.05 were considered statistically significant.

RESULTS

The inhibitory effect of L7G on PDGF-BB-induced VSMC proliferation

To evaluate the effect of L7G on the PDGF-BB-induced VSMC proliferation, we assessed the cell numbers after treating different concentrations of L7G on the PDGF-BB-stimulated VSMCs. As shown in Fig. 1, the number of cells was significantly increased after 50 ng/mL PDGF-BB-treatment (65.66 ± 5.66 × 10^4 cells/well), as compared to the non-stimulated group (43.16 ± 1.64 × 10^4 cells/well), and the increased cells were significantly reduced to 55.41 ± 0.92 × 10^4 cells/well after treatment of L7G at a concentration of 50 μM. In addition, the treatment of L7G (50 μM) for 24 h did not show any cytotoxicity to the VSMCs (data not shown); this indicated that the antiproliferative effect of the L7G on the VSMCs was not the result of cytotoxicity.

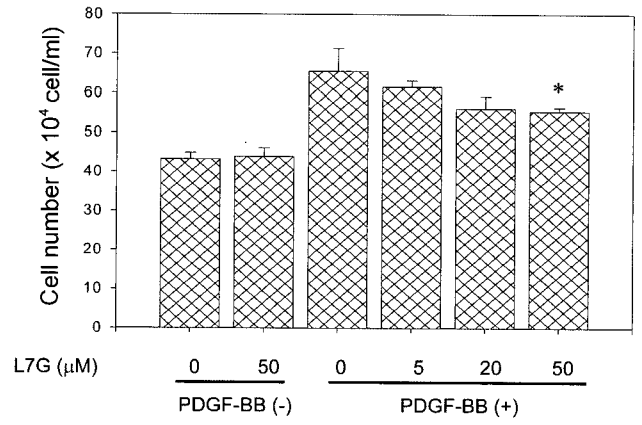

Fig. 1. Effects of L7G on the proliferation induced by PDGF-BB in VSMCs. The VSMCs were pre-cultured in serum-free medium in the presence or absence of the indicated concentration of L7G for 24 h, and then they were stimulated with 50 ng/mL PDGF-BB. The cells were trypsinized and then counted after 24 h with using a hemocytometer. The data are expressed as means ± S.E.Ms. (n = 4); *P < 0.05 compared with the PDGF-BB treatment alone.

Inhibitory effect of L7G on the PDGF-BB-induced [^{3}H]-thymidine incorporation

The effect of L7G on DNA synthesis was also assayed by using [^{3}H]-thymidine incorporation. As shown in Fig. 2, 50 ng/mL of PDGF-BB potently increased the [^{3}H]-thymidine incorporation from 52 to 1620 cpm/μg protein. The L7G significantly inhibited the PDGF-BB-induced DNA synthesis in a concentration-dependent manner. The inhibition percentages were 10.6, 23.8, and 28.0% at the concentrations of 5, 20, and 50 μM, respectively.

Effects of L7G on the ERK1/2, Akt and PLC-γ1 phosphorylations of the PDGF-BB-induced VSMCs

To examine the underlying mechanism of the antiproliferative effect that was exerted by L7G, we tested whether L7G affects the activation of the MAP kinases (ERK1/2), in view of their importance in the regulation of VSMC proliferation. L7G significantly inhibited the PDGF-BB-induced ERK1/2 phosphorylation by 51.5% ± 5.8% at a concentration of 50 μM (Fig. 3). Pre-treatment with L7G also significantly inhibited the PDGF-BB-induced Akt phosphorylation by 42.4% ± 4.1% at a concentration of 50 μM (Fig. 4). Similarly, the PDGF-BB-induced PLC-γ1 phosphorylation was decreased to 8.7% ± 3.4%, 27.8% ± 7.1%, and 48.6% ± 3.2% at concentrations of 5, 20, and 50 μM, respectively (Fig. 5). U0126 (ERK1/2 inhibitor; 10 μM), LY294002 (Akt inhibitor; 50 μM), and U73122 (PLC

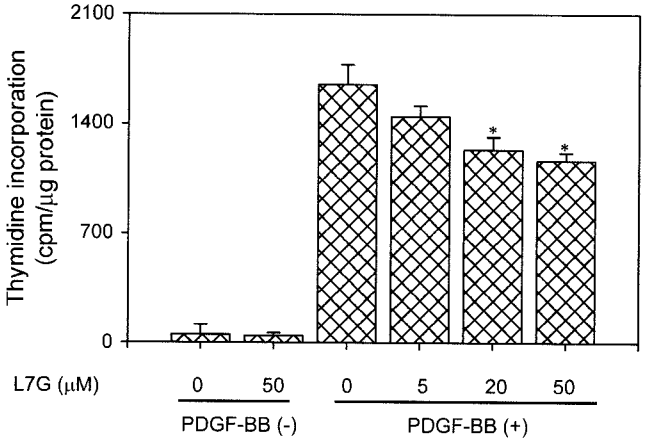

Fig. 2. The effect of L7G on the DNA synthesis induced by PDGF-BB in the VSMCs. The VSMCs were pre-cultured in serum-free medium in the presence or absence of the indicated concentration of L7G for 24 h, and then they were stimulated with 50 ng/mL PDGF-BB for 20 h. [^{3}H]-thymidine (2 μCi/mL) was added to the medium and the cells were incubated for 4 h. The radioactivity was determined by a liquid scintillation counter. The data are expressed as means ± S.E.Ms. (n = 4); *P < 0.05 compared with the PDGF-BB treatment alone.

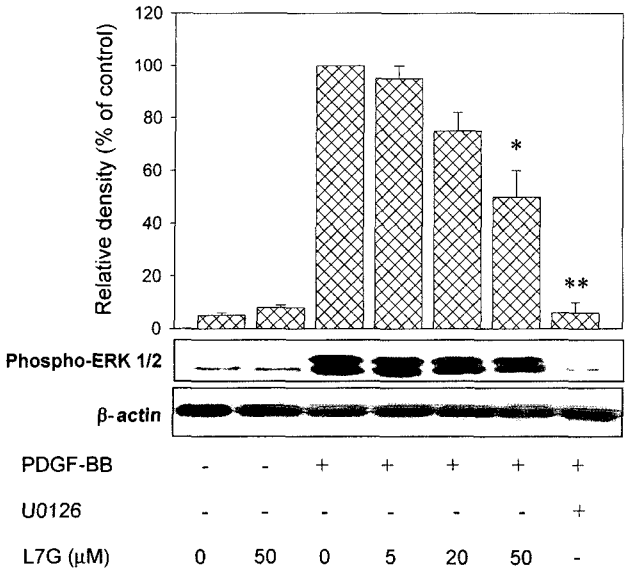

Fig. 3. Effect of L7G on the phosphorylation of ERK1/2 in the PDGF-BB-stimulated VSMCs. The confluent cells were pre-cultured in the presence or absence of the indicated concentration of L7G or U0126 (an ERK1/2 inhibitor, 10 μM) in serum-free medium for 24 h. The cells were briefly stimulated with 50 ng/mL PDGF-BB for 5 min. The cells were lysed, and the quantitative proteins were analyzed using 7.5-10% SDS–PAGE and by performing immunoblotting. After densitometric quantification, the data were expressed as means ± S.E.Ms. (n = 3); *P < 0.05, **P < 0.01 compared with the PDGF-BB treatment alone.

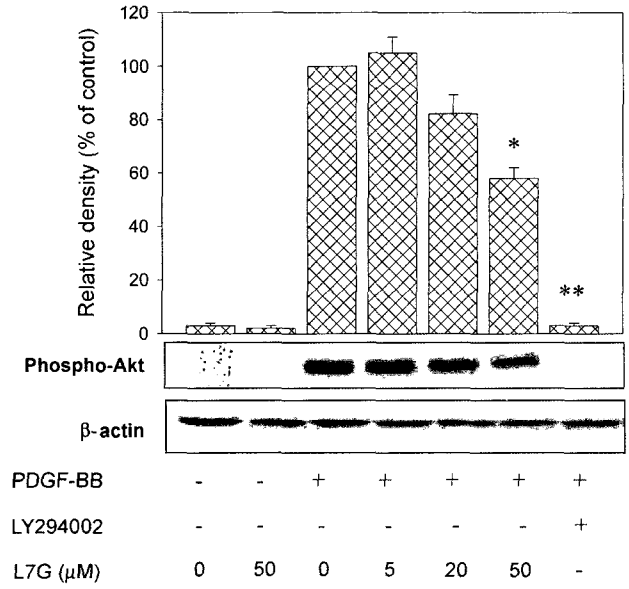

Fig. 4. Effect of L7G on the phosphorylation of Akt in the PDGF-BB-stimulated VSMCs. The confluent cells were pre-cultured in the presence or absence of the indicated concentration of L7G or LY294002 (an Akt inhibitor, 50 μM) in serum-free medium for 24 h. The cells were briefly stimulated with 50 ng/mL PDGF-BB for 15 min. The cells were lysed, and the quantitative proteins were analyzed using 7.5-10% SDS-PAGE and by performing immunoblotting. After densitometric quantification, the data were expressed as means ± S.E.Ms. (n = 3); *P < 0.05, **P < 0.01 compared with the PDGF-BB treatment alone.

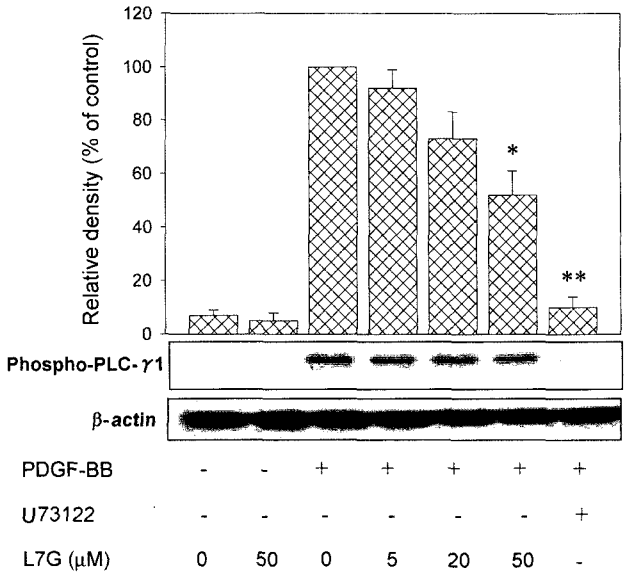

Fig. 5. Effect of L7G on the phosphorylation of PLC-γ1 in the PDGF-BB-stimulated VSMCs. The confluent cells were pre-cultured in the presence or absence of the indicated concentration of L7G or U73122 (a PLC inhibitor, 10 μM) in serum-free medium for 24 h. The cells were briefly stimulated with 50 ng/mL PDGF-BB for 5 min. The cells were lysed, and the quantitative proteins were analyzed using 7.5-10% SDS–PAGE and by performing immunoblotting. After densitometric quantification, the data were expressed as means ± S.E.Ms. (n = 3); *P < 0.05, **P < 0.01 compared with the PDGF-BB treatment alone.

inhibitor; 10 μM) were used as the positive controls, and they also significantly inhibited the ERK1/2, Akt and PLC-γ1 phosphorylation, respectively.

Effect of L7G on the phosphorylation of PDGF-receptor beta chain tyrosine kinase in the VSMCs

L7G was shown to inhibit the downstream components of PDGF-BB, such as ERK1/2, Akt and PLC-γ1 phosphorylation in a similar pattern. Thus, PDGF-receptor beta chain phosphorylation, which is upstream of PDGF-BB signaling, may be a direct target for L7G and so lead to the inhibition of VSMCs' proliferation. AG1295 at 20 μM, which is a positive control and a selective inhibitor of PDGF-receptor, significantly inhibited the PDGF-BB induced phosphorylation. However, in the presence of various concentrations of L7G, the PDGF-receptor beta chain phosphorylation was not altered (Fig. 6).

DISCUSSION

In the present study, we investigated the antiproliferative effect of L7G on VSMCs as an antiproliferating agent for employing it as a potential preventive/therapeutic agent to treat cardiovascular disease, including atherosclerosis, and we found that L7G inhibited the rat aortic VSMCs' proliferation and DNA synthesis that were observed in

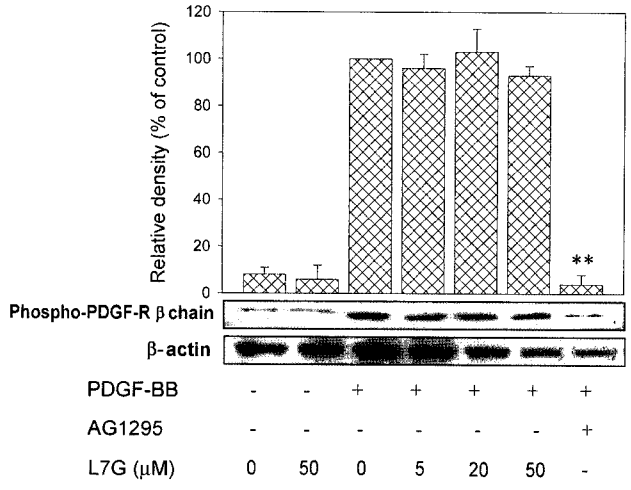

Fig. 6. The effect of L7G on PDGF- beta receptor phosphorylation in the VSMCs. The confluent cells were pre-cultured in the presence or absence of the indicated concentrations of L7G or AG1295 (20 μM) in serum-free medium for 24 h, and then they were briefly stimulated briefly by 50 ng/mL PDGF-BB at 37°C for 1 min. The cells were lysed, and the quantitative proteins were analyzed using 7.5% SDS-PAGE and by performing immunoblotting. The relative activities were quantified by scanning densitometry and this showed the levels of each activity as the relative value of the total PDGF-receptor. Western blotting was repeated three times. The data were expressed as means ± S.E.Ms. (n = 3).

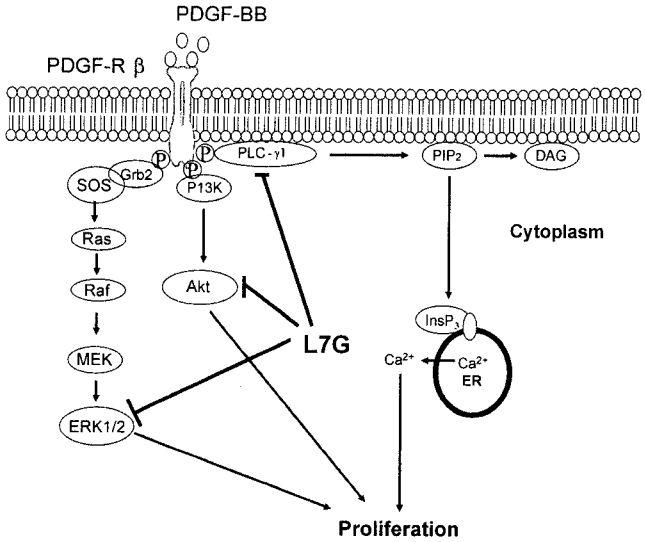

Fig. 7. A model for the inhibitory effect of L7G on the PDGF-BB-induced proliferation of vascular smooth muscle cells

response to PDGF-BB (Fig. 1 and 2). The inhibitory effect of L7G on the incorporation of [^{3}H]-thymidine into the cells gradually declined with the increased delay between the treatments of the VSMCs with luteolin and PDGF-BB. In addition, the antiproliferative effect of L7G on the VSMCs was not due to cellular cytotoxicity or apoptosis, which was demonstrated by the MTT assay and the flow cytometry assay (data not shown). Therefore, we hypothesized that the antiproliferative properties of L7G were causally related to the modulation of the signaling cascade involved in cell proliferation.

Several recent studies have emphasized the role of intracellular mitogenic signal transduction for cell growth, and the studies have also suggested the value of the transduction inhibitors. MAP kinase is an important player in the early intracellular mitogenic signal transduction for cell growth. Among the member of the MAP kinase family, ERK1/2 has been implicated in the proliferation of various type cells (Graf *et al.*, 1997). Therefore, we examined whether the anti-proliferation of L7G could act through the down regulation of the ERK1/2 cascade, and we found that L7G inhibited the PDGF-BB-induced ERK1/2 activation (Fig. 3). This data has shown that blocking of the ERK1/2 signal pathway may be important for the antiproliferative activity of L7G. In addition, we also determined the levels of phosphorylated PLC-γ1 and Akt, both of which were also activated by PDGF-BB. As shown in Fig. 4 and 5, L7G caused a marked decrease in the PDGF-BB-induced phosphorylation of PLC-γ1 and Akt with the same pattern being noted as was seen for the inhibition of ERK1/2 phosphorylation. These data indicated that the PDGF-receptor beta chain might be a potential target for L7G. However, the PDGF-receptor beta chain phosphorylation was not inhibited by L7G (Fig. 6). Ohlstein *et al.* have reported that a carvedilol compound inhibited VSMCs' proliferation, which was due to the inhibition of PDGF-Rβ tyrosine phosphorylation and its downstream intracellular signal transductions (Ohlstein *et al.*, 1993). The precise mechanism in response to the inhibition by L7G remains unknown. A further study to reveal this molecular mechanism is now in preparation.

Taken together, these results show that L7G inhibits the proliferation and DNA synthesis of VSMCs induced by PDGF-BB, and this is mediated by the inhibition of PLC-γ1, Akt and ERK1/2 phosphorylation. This beneficial property of L7G may be important for the therapeutic prevention/treatment of cardiovascular diseases.

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