

*Full Paper***An Inhibitory Effect of Chrysoeriol on Platelet-Derived Growth Factor (PDGF)-Induced Proliferation and PDGF Receptor Signaling in Human Aortic Smooth Muscle Cells**

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Abstract. Platelet-derived growth factor (PDGF)-BB is one of the most potent factors in the development and progression of various vascular disorders such as restenosis and atherosclerosis. Chrysoeriol is a flavonoid with antioxidant and anti-inflammatory activities. In this study, we investigated the effect of chrysoeriol on the proliferation of human aortic smooth muscle cells (HASMC). Chrysoeriol significantly inhibited PDGF (20 ng/mL)-induced migration and [³H]-thymidine incorporation into DNA at concentrations of 5 and 10 μ M without any cytotoxicity. Chrysoeriol also blocked PDGF-stimulated dissociation of actin filament and inhibited PDGF beta-receptor (R β) phosphorylation in a concentration-dependent manner. As a result, the downstream signal transduction pathways of PDGF-R β , including ERK1/2, p38, and Akt phosphorylation, were also inhibited by chrysoeriol in the same pattern. These findings suggest that in addition to its antioxidant and anti-inflammatory activities, chrysoeriol may be used for the prevention and treatment of vascular diseases and during restenosis after coronary angioplasty.

Keywords: smooth muscle cell, chrysoeriol, platelet-derived growth factor (PDGF)-induced proliferation, antiproliferative, migration

Introduction

Platelet-derived growth factor (PDGF)-BB is a potent chemoattractant produced by activated platelets and lesion macrophages that induces rapid downregulation of smooth muscle-selective markers such as α -actin in cultured smooth muscle cells (SMCs), and stimulates SMC proliferation and migration in arterial injury models (1). Importantly, growth arrest of proliferative/synthetic SMCs in culture restores the cells to a contractile phenotype. The fact that the growth arrest suppressed the pathologic phenotype of aortic SMCs has encouraged investigators to search for pharmacologic agents that inhibit aortic SMC growth (2). In addition to its mitogenic effects on SMCs, PDGF-BB regulates the expression of muscle-specific genes such as the smooth muscle

isoform of α -actin (SM- α -actin). PDGF-BB has been reported to decrease basal steady-state levels of SM- α -actin protein and block the increases of SM- α -actin protein caused by exposure to vasoconstrictors such as angiotensin II or arginine vasopressin (3).

PDGF-BB triggers the PI3-K/PKB (Akt) pathway in addition to the extracellular signal regulated kinase (ERK) and p38MAPK pathways. When the ERK and p38MAPK pathways were simultaneously blocked by their specific inhibitors or when an active form of either PI3-K or Akt was transfected, PDGF-BB in turn initiated the maintenance of the differentiated SMC phenotype. These findings with vascular SMCs indicate that the same signaling pathways might be involved in regulating the vascular SMC phenotype. These results suggest that changes in the balance between the PI3-K/Akt pathway and the ERK and p38MAPK pathways could determine the phenotypes of visceral and vascular SMCs (4). During vascular development, PDGF-BB stimulates pericyte migration or migration of other smooth muscle

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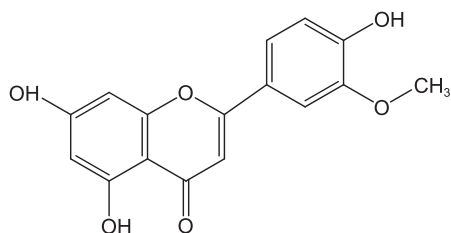


Fig. 1. Chemical structure of chrysoeriol.

precursors, which is necessary for correct vessel wall formation (5).

Chrysoeriol [5,7-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)chromen-4-one; Fig. 1] is a major yellow compound extracted from Rooibos tea (*Aspalathus linearis*) and has been used in traditional medicine to treat hyperactive gastrointestinal, respiratory, and cardiovascular disorders (6). Chrysoeriol has also been reported to have anti-inflammatory activity (7), anti-obesity activity (8), anti-oxidative activity (9, 10), and anti-mutagenic activity (11).

However, it is still largely unknown how chrysoeriol affects SMC function. In this study, we investigated the effect of chrysoeriol on PDGF-induced proliferation, migration, and signaling transduction pathways in HASMCs.

Materials and Methods

Cell cultures

HASMCs were purchased from Cascade Biologics, Inc. (Portland, OR, USA). HASMCs were grown in DMEM medium with growth supplement at 37°C in a humidified 95% air / 5% CO₂ atmosphere. Cells were used at passages three through eight. For all experiments, HASMCs were grown to 80%–90% confluence and made quiescent by starvation for at least 24 h. Chrysoeriol was purchased from Summit Pharmaceuticals International Corporation (Tokyo) (STOCK1N-14981).

[³H]-Thymidine incorporation assay

Cell proliferation was determined by [³H]-thymidine incorporation. Cells were incubated for 20 h with or without PDGF-BB (20 ng/mL) and various concentrations of chrysoeriol and then pulse-labeled with 1 μCi/mL of [³H]-thymidine for 4 h. Cells were harvested using a Universal Harvester (Perkin Elmer, Waltham, MA, USA), and then transferred to a GF/C filter (Perkin Elmer). The filter was dried and counted in scintillation fluid using a Microplate Scintillation and Luminescence Counter-Topcount NXT (Perkin Elmer).

Cell viability

Cell viability was determined with the trypan blue dye exclusion method. Cells were incubated for 24 h with or without PDGF-BB (20 ng/mL) and various concentrations of chrysoeriol and were then harvested from the dishes using a 0.1% w/v trypsin solution. Cell viability was examined by the trypan blue dye exclusion test. The number of viable cells was estimated by microscopic cell counting using a hemocytometer.

Cell migration assay

Modified Boyden transwell chambers were used for monitoring cell migration as previously described (12). Cells (35,000 cells/well) were seeded onto the upper chamber of the transwell, and the lower chamber contained the experimental reagents. Cells were allowed to migrate for 24 h, after which the inserts were removed. Nonmigrating cells in the upper chamber were removed, and cells in the bottom membrane were stained with 4 μg/mL calcein AM for 1 h. The number of migrated cells was determined by counting the number of stained cells from four randomly chosen high-power (×100) fields with a fluorescence microscope, at 485 nm excitation and 530 nm emission wavelengths.

Staining of F-actin with rhodamine phalloidin (12)

HASMCs were grown in Nunc chamber slides until about 60% confluence. After serum deprivation for 24 h, cells were incubated at 37°C for 1 h with the DMEM with or without chrysoeriol (10 μM) in the presence or absence of PDGF-BB (20 ng/mL). Cells were fixed and rendered permeable with 3.7% formaldehyde / 2% Triton X-100, washed with phosphate-buffered saline (containing 1% BSA), incubated with rhodamine phalloidin (13), and examined by fluorescence microscopy (1X71; OLYMPUS, Tokyo).

SDS-PAGE and immunoblotting

Western blotting for protein analysis was performed as described previously (14). Cells were harvested in lysis buffer containing 1 μM sodium vanadate, 1 μM phenylmethylsulfonyl fluoride, 5 μg/mL aprotinin, and 5 μg/mL leupeptin. The protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA). Lysates corresponding to equal amounts of proteins were boiled in Laemmli sample buffer, and the supernatants were loaded onto gels for SDS-PAGE. Proteins were transferred onto PVDF membranes and probed with the following primary antibodies: anti-phospho-PDGF-Rβ (1:1000), antiphospho-ERK1/2 (1:3000), anti-ERK (1:5000), and anti-phospho Akt (Thr308). All antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Appro-

appropriate horseradish peroxidase-coupled secondary antibodies were used at 1:10,000. Immunoreactive bands were visualized using enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, UK). The detected proteins were normalized to alpha-actin or the respective total protein as appropriate. The intensities of bands were quantified using Sicon-Image for Windows (Scion Corporation, Frederick, MA, USA).

Results

Effect of chrysoeriol on PDGF-stimulated HASMC proliferation

In the [^3H]-thymidine incorporation assay (Fig. 2A), stimulation with PDGF-BB (20 ng/mL) increased cell proliferation by about 5-fold. Chrysoeriol (1–10 μM) inhibited PDGF-stimulated cell proliferation in a concentration-dependent manner with 70% inhibition observed at 10 μM . When quiescent cells were treated with chrysoeriol (1–10 μM) for 24 h in the absence of PDGF-BB, no significant difference was observed in the extent of [^3H]-thymidine incorporation, suggesting that chrysoeriol is not cytotoxic at the concentrations tested. The lack of cytotoxicity of chrysoeriol at the concentrations used in these experiments was also ascertained by the trypan blue exclusion assay (Fig. 2B). The number of

cells was significantly increased after 20 ng/mL PDGF stimulation ($30.2 \pm 3.5 \times 10^4$ cells/well) compared with the non-stimulated group ($12.4 \pm 1.2 \times 10^4$ cells/well), and the increased cells were significantly reduced to 22.6 ± 3.6 , 14.8 ± 1.6 and $12.6 \pm 1.6 \times 10^4$ cells/well at concentrations of 1, 5, and 10 μM , respectively (Fig. 2B). Chrysoeriol treatment at 10 μM for 24 h did not show any cytotoxicity in the HASMCs (data not shown), indicating that the antiproliferative effect of chrysoeriol on HASMCs was not a result of cytotoxicity.

Chrysoeriol inhibits PDGF-stimulated HASMC migration

To investigate the effect of chrysoeriol on HASMC migration, cells were treated for 24 h with or without the indicated concentration of chrysoeriol (0–10 μM) in the Boyden chamber. The cells that migrated through the polystyrene-membrane with 8- μm pores were stained with calcein-AM and then counted with a fluorescence microscope. As shown in Fig. 3A, the cells that had migrated to the lower chamber were also visualized under a fluorescence microscope. PDGF-BB caused a 2-fold increase in migration of HASMC after 24 h (Fig. 3B), which was blocked by chrysoeriol. Treatment with chrysoeriol (1–10 μM) resulted in concentration-dependent inhibition of cell migration with statistical significance achieved at 10 μM ($P < 0.001$).

Chrysoeriol inhibits PDGF-stimulated cytoskeletal reorganization

As shown in Fig. 4, the actin cytoskeleton of HASMCs is also altered in response to PDGF-BB treatment (20 ng/mL). Untreated HASMCs contain multiple actin stress fibers, mostly oriented along the length of the cell body. With PDGF treatment, the cells appear to lose most of the actin stress fibers. These changes are consistent with cytoskeletal rearrangements that occur during migratory responses (15). Chrysoeriol treatment alone did not appear to alter the actin fibers. However, pretreatment with chrysoeriol (10 μM) inhibited most of the actin rearrangement of PDGF-BB.

Chrysoeriol attenuates PDGF-stimulated phosphorylation of PDGF-receptor β , ERK1/2, p38, and Akt

As shown in Fig. 5A, PDGF-BB markedly increased phosphotyrosine levels on the PDGF-R β , whereas chrysoeriol blocked this effect in a concentration-dependent manner. Neither treatment with PDGF nor chrysoeriol changed the protein levels of the PDGF receptor. In a similar manner, chrysoeriol blocked the capacity of PDGF-BB to stimulate the phosphorylation of downstream effectors ERK1/2 (Fig. 5B), p38 (Fig. 5C), and Akt (Fig. 5D).

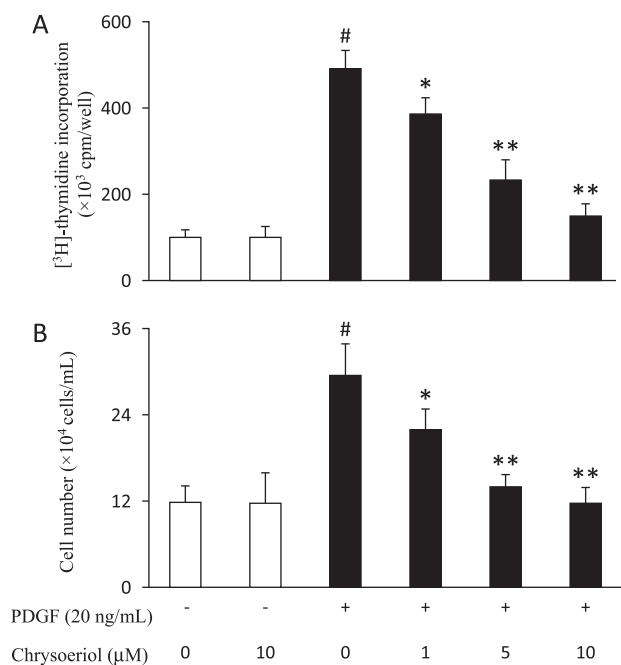


Fig. 2. The effect of chrysoeriol on PDGF-induced HASMC proliferation. Cells were incubated for 20 h with or without PDGF-BB and various concentrations of chrysoeriol, and then pulse-labeled with [^3H]-thymidine for 4 h (A). B: Cells were trypsinized and then counted using a hemocytometer. Results are each the mean \pm S.E.M. from three independent experiments. [#] $P < 0.001$, compared with the control; ^{*} $P < 0.05$ and ^{**} $P < 0.01$, compared with PDGF-stimulation.

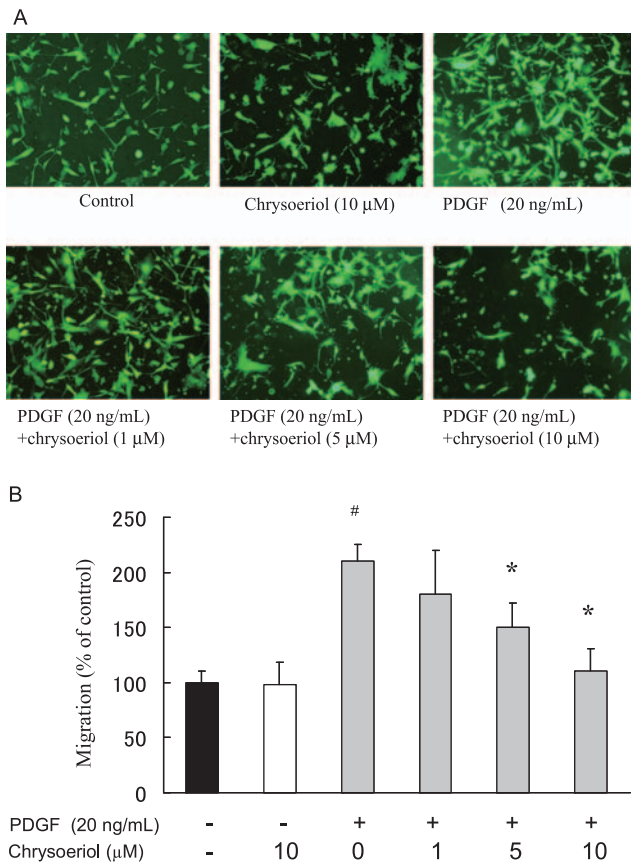


Fig. 3. Chrysoeriol attenuates PDGF-stimulated smooth muscle cell motility in a transwell migration assay. Treatment with chrysoeriol significantly reduced the number of cell migration in response to PDGF-BB. Results are each the mean \pm S.E.M. from three independent experiments. [#] $P < 0.005$, compared with the control; ^{*} $P < 0.001$, compared with PDGF-stimulation.

Discussion

PDGF binds to its cognate receptor tyrosine kinase, inducing autophosphorylation of tyrosine residues within the cytoplasmic domain of the receptors. This results in the recruitment and activation of specific signaling molecules that may mediate the migration and proliferation of VSMCs in response to injury (16). It has been suggested that different signaling pathways regulate the proliferative compared with the migratory responses to PDGF, with MAPK regulating proliferation and PI3K regulating migration (17). The ability of chrysoeriol to inhibit PDGF-stimulated ERK, p38, and Akt phosphorylation suggests that chrysoeriol may mediate its effects on PDGF signaling by acting upstream of the nuclear events.

In the present study, we found that chrysoeriol inhibited HASMC proliferation and DNA synthesis in response to PDGF-BB (Fig. 2A). The inhibitory effect of chrysoeriol on the incorporation of [³H]-thymidine

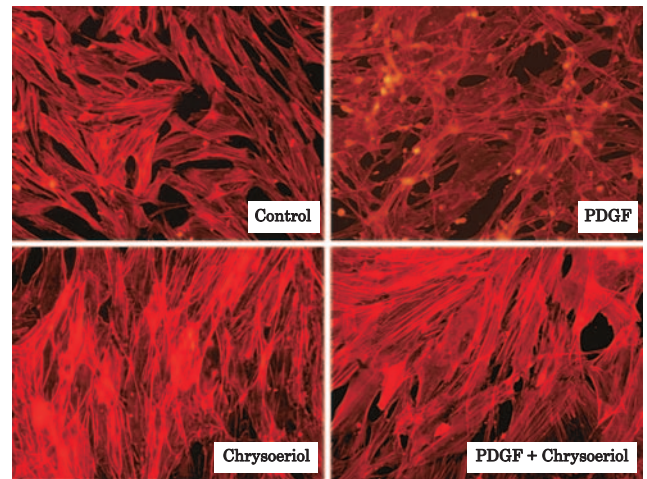


Fig. 4. Chrysoeriol inhibits PDGF-induced dissociation of actin microfilaments. Cells grown on slides were incubated with medium with or without chrysoeriol (10 μ M) in the presence or absence of PDGF-BB (20 ng/mL). After fixing and permeabilization, cells were stained with rhodamine phalloidin and examined by fluorescence microscopy.

into the cells gradually declined with the delay between the treatment of HASMCs with chrysoeriol and PDGF-BB. In addition, the antiproliferative effect of chrysoeriol on HASMC was not due to cellular cytotoxicity, as demonstrated by the cell counting (Fig. 2B) and MTT assays (data not shown).

Actin filament disassembly and assembly play important roles in the leading edge of a cell migrating in a gradient of PDGF-BB, where these processes enable cytoplasmic flow and protrusion of new leading lamellae (18). During chemotaxis of a cell in a gradient of a chemoattractant, intracellular signal transduction must be localized and signaling molecule gradients are crucial (19–21). Thus, the local intracellular balance and cycling between actin filament disassembly and assembly must be finely regulated (22, 23). Chrysoeriol also inhibits HASMC migration as well as MAPK activation and PDGF-induced dissociation of actin filament (Fig. 4).

MAPK plays an important role in early intracellular mitogenic signal transduction for cell growth. Among the members of the MAPK family, ERK1/2 has been implicated in proliferation of various cell types (24). Therefore, we examined whether the anti-proliferative effect of chrysoeriol could act via down regulation of the ERK1/2 signaling cascade. As shown in Fig. 5, chrysoeriol inhibited PDGF-BB-induced ERK1/2 activation. These data indicate that blocking the ERK1/2 signaling pathway may be important in the antiproliferative activity of chrysoeriol. In addition, we also determined the level of phosphorylated Akt, which was also

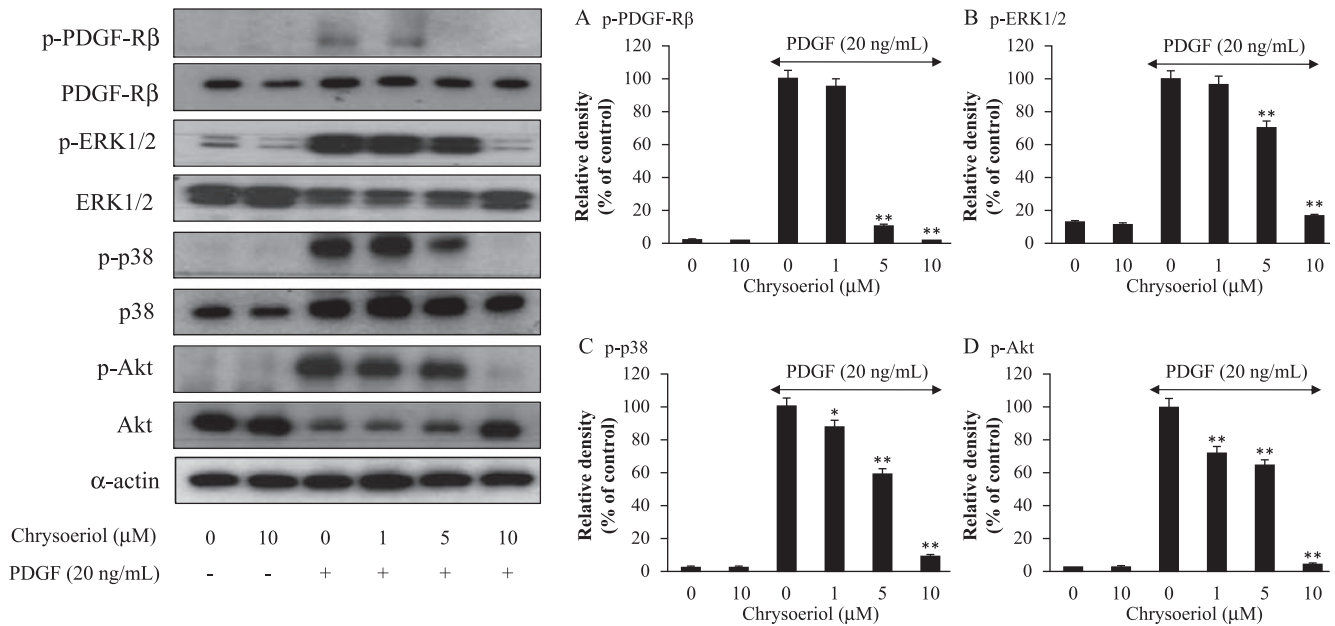


Fig. 5. Treatment with chrysoeriol inhibits PDGF-stimulated phosphorylation of PDGF-R β , ERK1/2, p38, and Akt. Cells were left untreated or treated without various concentrations of chrysoeriol (0 to 10 μ M) for 30 min in the presence or absence of PDGF (20 ng/mL) for 10 min. Cells were lysed, and lysates were immunoblotted with antibodies. The total protein in each lane was used for normalization, respectively. After densitometric quantification, data were each expressed as the mean \pm S.E.M. from three independent experiments. A: phospho-PDGF-R β , B: phospho-ERK1/2, C: phosphor-p38, and D: phosphor-Akt. * $P < 0.05$ and ** $P < 0.01$, compared with PDGF-stimulation.

activated by PDGF-BB. Chrysoeriol elicited a marked decrease in the PDGF-BB-induced phosphorylation of Akt in a pattern similar to chrysoeriol inhibition of ERK1/2 phosphorylation (Fig. 5). In this study, ERK1/2, p38, and Akt were used as a control for protein loading. However, the total amount of Akt rapidly decreased upon its activation. PDGF caused a rapid decrease in the Akt protein levels concomitant with Akt activation. PDGF causes the regulated proteolytic down-regulation of Akt, which is dependent on PI3K and proteasome activities. The proteasome-dependent down-regulation of Akt might be a fundamental mechanism to regulate the activity and function of Akt in VSMCs (25). These data indicate that PDGF-R β might be a potential target of chrysoeriol. In fact, chrysoeriol inhibited PDGF-R β phosphorylation in a concentration-dependent manner (Fig. 5). These results are also consistent with other studies showing that luteolin inhibited VSMC proliferation, which was due to the inhibition of PDGF-R β tyrosine phosphorylation and its downstream intracellular signal transduction (26).

In summary, these observations suggest that chrysoeriol inhibits PDGF-induced HASMC proliferation and migration through inhibition of PDGF-R β phosphorylation and downstream signal transduction such as ERK1/2 and Akt phosphorylation. In addition, chrysoeriol also blocked PDGF-stimulated dissociation of actin

filament. Therefore, chrysoeriol may be used for the prevention and treatment of vascular diseases or in restenosis after coronary angioplasty.

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