Phosphoinositide 3-kinase is a novel target of piceatannol for inhibiting PDGF-BB-induced proliferation and migration in human aortic smooth muscle cells

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Aims Abnormal migration and proliferation of human aortic smooth muscle cells (HASMCs) to the intima causes intimal thickening of the aorta, which is strongly related to the development of atherosclerosis. Previous studies have suggested that red wine polyphenols, particularly resveratrol, have great protective effects against cardiovascular diseases. Here, we compared the anti-atherosclerotic effect of piceatannol, a metabolite of resveratrol, and its underlying mechanisms.

Methods and results We demonstrated that piceatannol inhibited platelet-derived growth factor (PDGF)-BB-induced cell migration using a modified Boyden chamber assay and wound healing assay. Western blot analysis showed that PDGF-BB-induced phosphorylation of Akt, p70S6K, and p38 was inhibited by piceatannol, but not resveratrol. In vitro and ex vivo phosphoinositide 3-kinase (PI3K) assays demonstrated that piceatannol suppressed PI3K activity more effectively than resveratrol. PDGF-BB-induced migration and proliferation of HASMCs were inhibited by treatment with a commercial PI3K inhibitor, LY294002. Both in vitro and ex vivo pull-down assays revealed that piceatannol directly binds with sepharose 4B-PI3K beads in an ATP-competitive manner.

Conclusion The results of the present study demonstrate that piceatannol directly binds with PI3K in an ATP-competitive manner and suppresses PI3K activity with anti-atherosclerotic effects.

Keywords Phosphoinositide 3-kinase • Piceatannol • Platelet-derived growth factor • Migration • Atherosclerosis

1. Introduction

Atherosclerosis is a chronic inflammatory disease and a major cause of death in developed countries, both on its own and with complications such as myocardial infarction or stroke.† Excessive migration of smooth muscle cells (SMCs) from the media to the intima in arteries and their subsequent proliferation contribute to the development of atherosclerotic lesions. As a response to injuries or chronic inflammation, macrophages and T-lymphocytes are recruited to restore the immune system, and this series of inflammatory responses stimulates the migration and proliferation of SMCs.‡ SMCs in the medial layer of the vessel have contractile characteristics.§ After migrating to the intima, however, SMCs lose their original function of controlling the contraction of blood vessels and accumulate in the inner vessel with lipid-laden macrophages; this ultimately leads to vessel

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occlusion, resulting in the development and progression of atherosclerosis. Development and progression of atherosclerosis are triggered by multiple cytokines and growth factors, such as platelet-derived growth factor (PDGF), a potent chemoattractant for SMCs composed of four different genes: PDGF-A, PDGF-B, PDGF-C, and PDGF-D. Among them, only PDGF-BB can bind with all homo- or heterodimer receptors (PDGFR-αα, PDGFR-αβ, and PDGFR-ββ). The binding of PDGF-BB to PDGFR-α or PDGFR-β chains leads to the phosphorylation of PDGFR and activates many Src homology-2 domain-containing molecules, including phosphoinositide 3-kinase (PI3K).

PI3K is a heterodimeric lipid kinase consisting of an 85-kDa regulatory subunit and a 110-kDa catalytic subunit that plays a pivotal role in cell movement, growth, and anti-apoptosis. It is involved in the proliferation of SMCs by various inducers, including PDGF-BB. Multiple lines of evidence have shown that the migration of SMCs requires the activation of the PI3K pathway. PI3K was reported to regulate cell migration and collagen contraction, which is thought to be a regulator of interstitial fluid pressure, based on the experiments on fibroblasts cultured from mutant embryos. Therefore, targeting the PI3K pathway may be a promising strategy in preventing the proliferation and migration of SMCs.

Despite the consumption of a high cholesterol-enriched diet, the French have relatively low mortality, known as the so-called 'French paradox'. Resveratrol (3,5,4'-trihydroxystilbene; Figure 1A) from red wine is considered to be an effective molecule in the prevention of cardiovascular diseases. Piceatannol (3,5,3',4'-tetrahydroxystilbene; Figure 1B) which was first isolated from the seeds of Euphorbia lagascae is a natural analogue of resveratrol. Piceatannol has beneficial effects in many major diseases, including cancer and neurodegenerative diseases. Piceatannol is well known as an inhibitor of spleen tyrosine kinase (Syk) and inhibits the migration of rat aortic smooth muscle cells (RASMCs) by attenuating p38 mitogen-activated protein kinase (MAPK)/HSP27 pathways. Previous studies have reported that piceatannol suppresses the tumour necrosis factor-α-induced proliferation of human myeloid cells by regulating NF-κB activation and inhibits the growth of colorectal cancer cells and arrested Caco-2 cells in the S phase of the cell cycle. Here we report...
that piceatannol is a natural inhibitor of PI3K and has a greater inhibitory effect than resveratrol in PDGF-BB-induced migration and proliferation in human aortic smooth muscle cells (HASMCs).

2. Methods

2.1 Chemicals

Piceatannol was obtained from AG Scientific (San Diego, CA, USA), and resveratrol and the antibodies against β-actin were purchased from Sigma-Aldrich (St Louis, MO, USA). LY294002 was acquired from Cal Biochem (San Diego, CA, USA) and PDGF-BB was supplied by R&D Systems (Minneapolis, MN, USA). MCDB131 medium, streptomycin/penicillin, insulin, foetal bovine serum (FBS), and L-glutamine were purchased from Gibco-BRL (Carlsbad, CA, USA), and epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) were obtained from Invitrogen (Carlsbad, CA, USA). The antibodies against phosphorylated Akt (Thr308 and Ser473) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); phosphorylated p70S6 kinase (p70S6K), total p70S6K and total Akt (pan) antibodies were purchased from Cell Signaling Biotechnology (Beverly, MA, USA); and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) was purchased from the USB Corporation (Cleveland, OH, USA). CNBr-Sepharose 4B was supplied by GE Healthcare (Buckinghamshire, UK) and protein G agarose was acquired from KPL Corporation (Gaithersburg, MD, USA). [γ-32P]ATP was purchased from Institute of Isotopes Co, Ltd (Budapest, Hungary), and the protein assay kit was obtained from Bio-Rad Laboratories (Hercules, CA, USA).

2.2 Cell culture

HASMCs originating from normal human tissue were obtained by permission according to the Declaration of Helsinki and supplied by BioWhittaker (Walkersville, MD, USA). They were cultured in monolayers at 37°C in a 5% CO2 incubator in MCDB131 containing 10% FBS, 2 mM L-glutamine, EGF, bFGF, insulin, and streptomycin/penicillin. All experiments were performed with HASMCs from passages 7 to 13.

2.3 Cell viability/proliferation assay

To estimate cell proliferation and viability, HASMCs were seeded onto 24-well plates (n = 3) and grown to near confluence in media containing 10% FBS. HASMCs were then starved for 24 h. The cells were either treated or not treated with different concentrations of resveratrol, piceatannol (5–20 μM), or LY294002 (20 μM) for 1 h, and then 10 ng/mL PDGF-BB was added for the proliferation assay, or only samples for the viability assay for 48 h. Next, 50 μL of MTT solution (0.5 mg/mL) was added to each well containing 500 μL of conditioned media and incubated for another 4 h at 37°C. Then, the medium was removed and 400 μL of dimethyl sulphoxide (DMSO) was added to each well. After shaking, 200 μL of DMSO from each well was transferred to a 96-well plate. The cell viability and rate of proliferation was determined by reading the absorbance at 570 nm, and results were expressed as the fold change in absorbance relative to the untreated control.

2.4 Wound healing migration assay

HASMCs (5 x 10^5) were plated on six-well dishes and grown to post-confluence in 3 mL of growth medium. The cells were subjected to injury using a 2-mm wide tip. They were then treated with 10 ng/mL PDGF-BB alone or together with resveratrol, piceatannol, or LY294002. The cells were allowed to migrate, and photographs were taken through an inverted microscope (x100 magnification).

2.5 Boyden chamber migration assay

The migration assay was performed using the Transwell system (Corning Costar, Cambridge, MA, USA). The lower side of the filter was coated with 10 μL of type collagen (Collaborative Research, Lexington, KY, USA). The lower compartment was filled with 600 μL of MCDB131 medium containing 0.1% bovine serum albumin and resveratrol, piceatannol or LY294002. Cultured cells (3 x 10^5) were resuspended in 100 μL of MCDB131 medium and placed in the upper part of the Transwell plate. Cells were incubated for 8 h in a humidified atmosphere of 5% CO2 at 37°C. HASMCs were fixed with methanol and stained with haematoxylin for 10 min followed by eosin. HASMCs from the upper surface of the filter were mechanically removed by wiping with a cotton swab, and the migrated cells were determined by counting the cells that migrated to the lower side of the filter using a microscope. Ten randomly selected fields were counted, and each sample was assayed in triplicate.

2.6 Western blotting

After HASMCs (1 x 10^4) were cultured in a 6 cm diameter dish for 48 h, they were starved in serum-free medium for another 24 h. The cells were then treated with piceatannol or resveratrol (5–20 μM), or various inhibitors (20 μM), for 1 h before exposure to 10 ng/mL PDGF-BB for 5–15 min. The harvested cells were disrupted, and the protein concentration was determined using a dye-binding protein assay kit (Bio-Rad Laboratories) according to the manufacturer’s instructions. Lysate protein was subjected to 10% SDS–PAGE and electrophoretically transferred to a polyvinylidene difluoride membrane (Pall Corporation, East Hills, NY, USA). After blotting, the membrane was incubated with the specific primary antibody at 4°C overnight. Protein bands were visualized with a chemiluminescence detection kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) after hybridization with a horseradish peroxidase-conjugated secondary antibody. Image J software was used for data analysis.

2.7 In vitro PI3K assay

The in vitro kinase assays were performed according to the manufacturer’s instructions. An active PI3K protein (100 ng) was incubated with resveratrol, piceatannol, or LY294002 at 30°C. Then, the mixtures were incubated with 20 μL of 0.5 mg/mL phosphatidylinositol (Avanti Polar Lipids, Alabaster, AL, USA) at room temperature, followed by incubation with the reaction buffer (Supplementary material online) for an additional 10 min at 30°C. The reaction was stopped by adding 15 μL of 4 N HCl and 130 μL of chloroform/methanol (1:1). After vortexing, 30 μL of the lower chloroform phase was spotted onto a 1% potassium oxalate-coated silica gel plate (Merck, Darmstadt, Germany), which was previously activated for 1 h at 110°C. The resulting [32P]-labelled PI3P was separated by thin layer chromatography, and the radio-labelled spots were visualized by autoradiography.

2.8 Ex vivo PI3K immunoprecipitation and kinase assay

HASMCs were grown to subconfluence in 15 cm plates and then serum-starved with FBS-free MCDB media for 24 h. Cells were pretreated in the presence or absence of resveratrol or piceatannol (5–20 μM) for 1 h, and then treated with 10 ng/mL PDGF-BB for 15 min. Afterward, the cells were lysed in 300 μL of PI3K assay lysis buffer (Supplementary material online), then centrifuged at 16,000 g for 10 min. The lysates containing 500 μg of protein were used for immunoprecipitation with a monoclonal PI3-kinase p110 antibody and were subsequently incubated with Protein A/G Sepharose beads. The immunoprecipitates were washed twice with washing buffers (Supplementary
material online). After the last wash, the immunoprecipitates were resuspended in 20 μL of buffer 3 (Supplementary material online) on ice for 5 min, and then 20 μL of 0.5 mg/mL phosphatidylinositol was added. After 5 min at room temperature, the immunoprecipitates were incubated with reaction buffer and the assay was performed as mentioned in the in vitro PI3K assay.

### 2.9 Ex vivo Syk immunoprecipitation and kinase assay

For the ex vivo Syk kinase assay, HASMCs were grown to subconfluence in 15 cm plates and then serum-starved with FBS-free MCDB media for 24 h. Cells were either treated or not treated with piceatannol (0–20 μM) for 1 h, and then treated with 10 ng/mL PDGF-BB and harvested after 15 min, disrupted with lysis buffer (Supplementary material online) and finally centrifuged at 16 000 × g for 10 min in a microcentrifuge. Lysate samples containing 500 μg of protein were used for immunoprecipitation with an antibody against Syk and incubated at 4°C overnight. Protein A/G Plus agarose beads were then added, and the mixture was rotated continuously for another 3 h at 4°C. The beads were washed three times with kinase buffer (Supplementary material online). Next, 2.5 μL of 1 mg/mL Syk substrate peptide were added with 10 μL of diluted [γ-32P]ATP in magnesium acetate–ATP cocktail buffer (2.5 mM HEPES (pH 7.4), 50 mM magnesium acetate, and 0.5 mM ATP). The reactions were incubated at 30°C for 10 min, and then 15 μL aliquots were transferred onto p81 paper and washed three times with 0.75% phosphoric acid for 5 min and once with acetone for 5 min. The radioactive incorporation was determined using a scintillation counter. Each experiment was performed three times.

### 2.10 Pull-down assay

PI3K (100 ng) was incubated with piceatannol–sepharose 4B (or Sepharose 4B alone, as a control) beads (100 μL, 50% slurry) in reaction buffer (Supplementary material online). After incubation with PDGF-BB for 8 h, Migrated cells were photographed (C). Migrated cells were counted as described in Methods section. (a) Untreated control; (b) PDGF-BB; (c) PDGF-BB and 20 μM resveratrol; (d) PDGF-BB and 20 μM piceatannol. Results are expressed as the number of migrated cells relative to untreated controls as determined from three-independent experiments (D). *p < 0.05 vs. control; **p < 0.05 vs. PDGF-BB; ***p < 0.01 vs. PDGF-BB.
gentle rocking overnight at 4°C, the beads were washed five times (Supplementary material online), and the proteins bound to the beads were analysed by immunoblotting.

2.11 ATP and piceatannol competition assay

Briefly, 100 ng of active PI3K was incubated with ATP (0, 10, or 100 μM) or piceatannol (0, 5, 10, or 20 μM) for overnight. After incubation, 100 μL piceatannol–sepharose 4B or sepharose 4B in reaction buffer (see Pull-down assay section above) or ATP-agarose for 2 h at 4°C. After incubation with gentle rocking overnight at 4°C, the beads were washed five times (Supplementary material online), and the proteins bound to the beads were analysed by immunoblotting.

2.12 Statistical analysis

Data are expressed as the mean ± SD values. One-way analysis of variance (ANOVA) was used for comparisons in the experiments with multiple time points and concentrations. When ANOVA indicated statistical significance, Duncan’s multiple range test was used to determine which means were significantly different. A probability value of $P < 0.05$ was used as the criterion for statistical significance.
3. Results

3.1 Piceatannol inhibits PDGF-BB-induced proliferation in HASMCs

The abnormal proliferation and accumulation of SMCs in the intima layer are prominent features of atherosclerosis.3 We examined the anti-proliferative effects of resveratrol and piceatannol, which were not cytotoxic to HASMCs at concentrations of 5, 10, or 20 μM (Figure 1C). Stimulation with PDGF-BB (10 ng/mL) for 48 h increased HASMCs proliferation by 1.8-fold (180 ± 16.9%) compared with the non-treated control. Pretreatment with piceatannol suppressed PDGF-BB-induced cell growth levels to that of the control, and resveratrol also attenuated PDGF-BB-induced cell growth by ~75% that of piceatannol at 20 μM (121 ± 7.9%). The inhibitory effects of piceatannol were much stronger than those of resveratrol (P < 0.01; Figure 1D).

3.2 Piceatannol suppresses PDGF-BB-induced migration in HASMCs

Migration of SMCs is a critical step in the growth of an atherosclerotic lesion.2–3 To evaluate the effects of resveratrol and piceatannol on the PDGF-BB-induced migration of HASMCs, we performed two kinds of migration assays. First, we examined the effects of resveratrol and piceatannol on cell migration using a wound healing migration assay. As expected, resveratrol inhibited PDGF-BB-induced migration. However, resveratrol could not block PDGF-BB-induced migration completely (Figure 2A), whereas the anti-migratory ability of piceatannol acted in a concentration-dependent manner; above 10 μM, piceatannol inhibited PDGF-BB-induced migration completely (Figure 2B). To confirm this result, we performed a modified Boyden chamber assay. Piceatannol markedly inhibited PDGF-BB-induced migration of HASMCs, whereas resveratrol only slightly inhibited it (Figure 2C and D).

3.3 Piceatannol represses PDGF-BB-induced phosphorylation of Akt, p70S6K, and MAPKs in HASMCs

Several studies have reported that the level of phosphorylated Akt and MAPKs is elevated in response to PDGF9,18–19 and that all three MAPKs participate in migration and proliferation of RASMCs.8 To elucidate the inhibitory mechanisms on migration and proliferation, we next investigated the effect of piceatannol on phosphorylation of Akt, the downstream p70S6K, and Akt in HASMCs. Treatment with 10 μM piceatannol reduced PDGF-BB-induced phosphorylations of Akt at both sites, Ser473 (Figure 3A) and Thr 308 (Figure 3B), and phosphorylation of p70S6K (Figure 3C). PDGF-BB-induced phosphorylation of p38 was inhibited by treatment with 20 μM of piceatannol (Figure 3D). Phosphorylation of c-Jun N-terminal kinase was decreased by piceatannol slightly, but this reduction is not statistically significant (Figure 3E). Piceatannol did not affect PDGF-BB-induced phosphorylation of ERK in HASMCs (Figure 3F), and up to 20 μM of resveratrol had no inhibitory effect on PDGF-BB-induced phosphorylation of Akt, p70S6K, and all three MAPKs (Figure 3).

3.4 Piceatannol attenuates PI3K in vitro and PDGF-BB-induced PI3K and Syk activity ex vivo

To investigate whether PI3K might be a molecular target of piceatannol in the inhibition of migration and proliferation in HASMCs, we next performed in vitro and ex vivo PI3K assays. Piceatannol strongly inhibited PI3K activity and its effect was superior to that of LY294002, a well-known PI3K inhibitor, at the same concentration (Figure 4A). Results from the ex vivo kinase using cell lysates were similar to those from the in vitro experiment.
(Figure 4B). These results suggest that the anti-migratory effect of piceatannol was due to the direct inhibition of PI3K activity. We also measured Syk activity in piceatannol-treated HASMCs, because piceatannol is well known for Syk inhibitor. Piceatannol inhibited Syk activity in PDGF-BB-induced HASMCs (Figure 4C).

3.5 Piceatannol directly binds with PI3K in vitro and ex vivo and competes with ATP for binding to PI3K

To determine whether piceatannol binds with PI3K directly, we performed in vitro and ex vivo pull-down assays. In the in vitro pull-down assay using the PI3K protein, PI3K was found in the piceatannol beads (Figure 5A, lane 3) but not in the control beads (Figure 5A, lane 2). PI3K protein was used as a control (Figure 5A, lane 1). We also observed binding between piceatannol and PI3K (Figure 5B, lane 3) in the ex vivo experiment using HASMC lysates. To examine whether piceatannol binds with PI3K in a competitive manner with ATP, we performed an ATP-competition assay. Binding between piceatannol and PI3K decreased with increasing amounts of ATP (Figure 5C). And interaction between PI3K and ATP also decreased by the addition of piceatannol (Figure 5D). These results suggest that piceatannol is competitive with ATP in suppressing PI3K activity.

3.6 The PI3K pathway is involved in PDGF-BB-induced migration and proliferation in HASMCs

We demonstrated that piceatannol dose dependently inhibited the PI3K/Akt pathway in PDGF-BB-treated cells. We next examined the specific role of the PI3K pathway in PDGF-BB-induced migration and proliferation in HASMCs using a specific inhibitor of PI3K, LY294002. LY294002 was not cytotoxic at 20 μM (Figure 1C). Treating HASMCs with LY294002 significantly reduced PDGF-BB-induced proliferation and migration to non-treated control levels (Figure 6).

4. Discussion

Several epidemiological studies have indicated that consumption of red wine and the incidence of coronary heart disease are inversely correlated. Resveratrol, a polyphenolic compound present in red wine, is considered an active compound with a cardioprotective effect.14–15 After entering the bloodstream, resveratrol is metabolized into a glucuronidated or sulphated form within 15 min, and moderate consumption of red wine is insufficient for resveratrol to reach an effective concentration.20 However, resveratrol generates piceatannol as a major metabolite through CYP1B1, a cytochrome p450 enzyme.21 Piceatannol has a chemical structure similar to that of resveratrol, with the addition of a hydroxyl group at the 3'-position in the B-ring moiety of resveratrol. Our previous studies demonstrated that small structural differences between quercetin and kaempferol, which differ by only one hydroxyl group at the 3'-position, resulted in different affinities to MEK, which is considered a molecular target in the inhibition of neoplastic transformation.22 Consistent with this point of view, minor differences between two chemicals are capable of causing different results under physiological conditions. In the present study, we demonstrated that piceatannol has significant inhibitory effects against the
PDGF-BB-induced proliferation and migration of SMCs, whereas resveratrol has relatively minor inhibitory effects.

PDGF-BB is considered the most prominent chemoattractant for SMCs, and it activates multiple signalling pathways, including PI3K/Akt and MAPKs.\(^23\)–\(^24\) We found that piceatannol inhibited phosphorylation of Akt and p38, but not ERK and JNK. The constitutively active p110-CA class IA PI3K stimulated human pulmonary vascular SMC proliferation and migration, whereas the dominant-negative p110-CA class IA PI3K inhibited PDGF-BB-induced DNA synthesis.\(^7\) Recent studies have demonstrated that the immune deletion of PI3K\(_y\) not only reduced atherosclerotic plaque size, but also promoted its stabilization in the LDLR\(^{-/}\) and ApoE\(^{-/}\) mice model.\(^25\) Plaque stabilization is associated with the prevention of plaque rupture, which causes thrombus formation.\(^25\) In our study, piceatannol exerted much stronger inhibitory effects on PI3K activity both in vitro and ex vivo than resveratrol. We confirmed that piceatannol directly binds with PI3K in an ATP competitive manner. Furthermore, treatment with LY294002 had similar inhibitory effects as that of piceatannol on PI3K pathways. Syk is required for PDGF-BB-induced proliferation and migration in SMCs through controlling p38.\(^9\) Piceatannol has been used as a Syk inhibitor.\(^26\) In this study, the PDGF-BB-induced Syk activity in SMCs was significantly inhibited by piceatannol treatment. Thus, inhibition of p38 phosphorylation by piceatannol might stem from inhibition of Syk activity. These results suggest that PI3K and Syk are potent molecular targets for inhibition of PDGF-induced proliferation and migration in SMCs by piceatannol.

After the clinical success of Gleevec, multiple target kinase inhibitors came into the spotlight. Even though multiple target kinase inhibitors have safety issues, they may have broader applications than kinase inhibitors with one specific target. Small molecules in food are generally regarded as safe because of their long-time use in food.\(^27\)–\(^28\) Therefore, elucidating a new target of piceatannol enlarges the application of this compound. In this study, we suggest that PI3K is a novel molecular target of piceatannol. X-ray crystallography and kinetic analysis are needed to

![Figure 6](https://example.com/figure6.png)
elucidate the structure and binding affinity of the piceatannol–PI3K complex in further study. In summary, piceatannol, an analogue of resveratrol, has a more potent inhibitory effect on PDGF-BB-induced HASMC migration and proliferation than resveratrol, and PI3K may be a novel molecular target of piceatannol in inhibiting the pathogenesis of atherosclerosis.

**Supplementary material**

Supplementary material is available at *Cardiovascular Research* online.

**Conflict of interest:** none declared.

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