Eupatilin, a Major Flavonoid of *Artemisia*, Attenuates Aortic Smooth Muscle Cell Proliferation and Migration by Inhibiting PI3K, MKK3/6, and MKK4 Activities

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Abstract

Eupatilin, a major flavonoid of plants in the genus *Artemisia*, has been shown to exhibit anti-inflammatory, anti-oxidative, and anti-tumor effects. However, the potential anti-atherogenic effects of eupatilin and any underlying mechanisms have not been investigated. In the present study, we sought to determine the effects of eupatilin on phenotypes induced by the growth factor PDGF-BB in human aortic smooth muscle cells. Here we show that aortic sprouting as well as PDGF-BB-induced proliferation and migration of human aortic smooth muscle cells were significantly inhibited by eupatilin. We found that eupatilin inhibited PI3K activity, causing a direct effect on phosphorylation of the downstream kinases Akt and p70S6K. In parallel, eupatilin also inhibited the phosphorylation of MKK3/6-p38 MAPK and the MKK4-JNK pathway. Moreover we found that eupatilin exhibited stronger inhibition effects on PDGF-BB-induced proliferation and migration of human aortic smooth muscle cells than PI3K, p38 MAPK, and JNK pathway inhibitors. Taken together, our results indicate that eupatilin is a potent anti-atherogenic agent that inhibits PDGF-BB-induced proliferation and migration in HASMCs as well as aortic sprouting, which is likely mediated through the attenuation of PI3K, MKK3/6, and MKK4 activation.

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

Introduction

Atherosclerosis is a chronic disease of the cardiovascular system and a major cause of death in the developed world [1]. Atherosclerotic development is characterized by inflammation and the subsequent thickening and hardening of blood vessel walls. This can lead to an interference with blood circulation and pressure which, if sustained, eventually causes secondary cardiovascular complications [2]. The chronic state of arterial inflammation increases the release of cytokines and growth factors, including platelet-derived growth factor (PDGF), which promote abnormal proliferation and migration of smooth muscle cells (SMCs) in the arterial media layer [3]. The excessive proliferation and migration of SMCs to the intima of arteries in turn contributes to abnormal hardening and thickening of the artery wall, resulting in a loss of contractile function [2]. If left unchecked, these activities result in dysfunction of the artery and the progression of atherosclerosis. Therefore, the prevention of these abnormalities constitutes a logical strategy for the prevention of atherosclerosis [2,4].

PDGF is a growth factor secreted by platelets that contributes to the development of atherosclerosis by inducing proliferation and migration of SMCs [1,5]. The PDGF receptor is a receptor tyrosine kinase. There are five different isoforms of PDGF: PDGF-AA, PDGF-BB, PDGF-CC, PDGF-DD, and the heterodimer form, PDGF-AB. Among these isoforms, PDGF-BB exhibits the most powerful signal transduction abilities and is the only one that binds all isoforms of the PDGF receptor [6]. Ligand-induced PDGFR activation subsequently activates various signaling intermediates, including phosphoinositide-3-kinase (PI3K)/Akt and mitogen-activated protein kinases (MAPKs), which play a crucial role in the proliferation and migration of cells [7,8].

PI3K is a heterodimeric lipid kinase that plays a pivotal role in cell movement, growth, and cell survival. The PI3K signaling pathway involves numerous downstream kinases, the most prominent of which are Akt and p70S6K [9,10]. Multi-

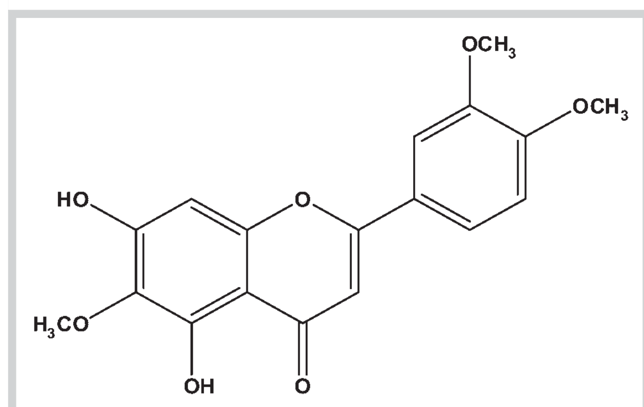
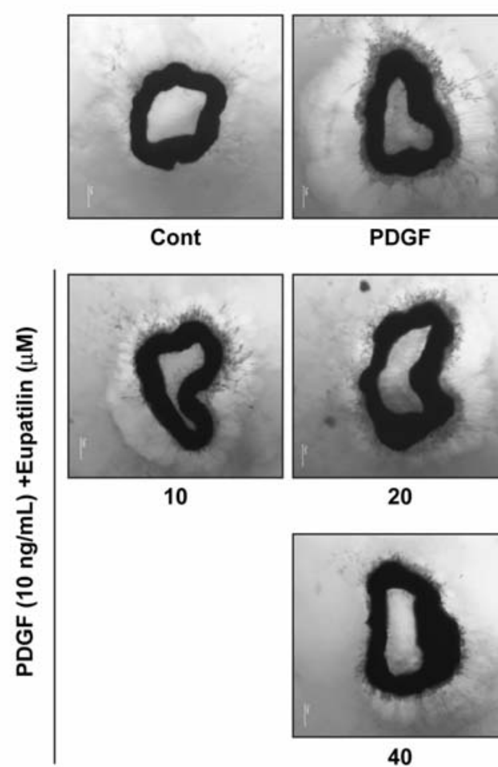


Fig. 1 Chemical structure of eupatilin. Eupatilin (2-(3,4-dimethoxyphenyl)-5,7-dihydroxy-6-methoxychromen-4-one) is an O-methylated flavone and a type of flavonoid found in *Artemisia asiatica*.

ple lines of evidence show that the proliferation and migration of SMCs require activation of the PI3K pathway [11–13]. The MAPK family is composed of serine/threonine protein kinases including the extracellular signal-regulated kinase (ERK) [14], p38-MAPK, and c-Jun N-terminal kinase (JNK). These are in turn regulated by upstream kinases (MAPKKs including MEK1/2, MKK3/6, and MKK4). The MAPK pathways play an important role in various biological functions including cell proliferation and migration [15, 16] and are activated by stimuli including PDGF, allowing for control of proliferation and migration of SMCs [17–19]. Therefore, targeting the PI3K/Akt and MAPKK/MAPK signaling pathways represents a promising strategy for preventing the proliferation and migration of SMCs and subsequent atherosclerotic development.

Evidence suggests that a diet rich in foods containing high concentrations of polyphenols, such as red wine, green tea, and fruits, confers various pharmacological benefits including antioxidant, anti-carcinogenic, and anti-atherogenic effects [20–22]. Although the flavonoid eupatilin (2-(3,4-dimethoxyphenyl)-5,7-dihydroxy-6-methoxychromen-4-one) derived from plants of the genus *Artemisia* (**Fig. 1**) is a well-established anti-inflammatory and anti-cancer agent [23–26], its potential inhibitory effects on atherosclerotic development remain unclear. In the present study, we analyzed the effects of eupatilin on PDGF-BB-induced proliferation and migration of human aortic smooth muscle cells (HASMCs) and examined the mechanisms responsible for them.



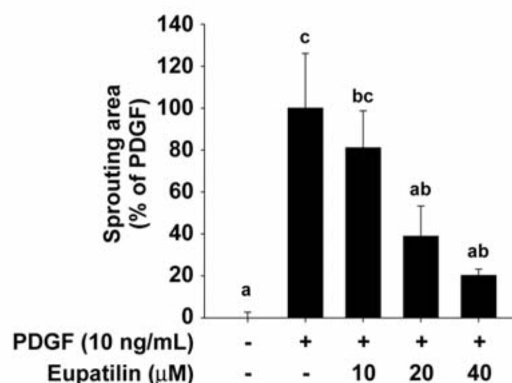


Fig. 2 Effects of eupatilin on PDGF-BB-induced sprout formation in rat aortic rings. **A,B** Aortic rings (1-mm) embedded and cultured in Matrigel were treated with PDGF-BB with 10, 20, or 40 µM of eupatilin as indicated. All images were obtained on day 5 of treatment. **A** Light microscopy images showing the effects of eupatilin on PDGF-BB-induced sprout formation in rat aortic rings and **B** quantification of sprouting areas as described in Materials and Methods. Data are represented as mean ± SEM values of three independent experiments. Means with letters (a–c) within the graph are significantly different from each other at $p < 0.05$.

Results

An abnormal expansion of the aortal media layer is a prominent feature of atherosclerosis [2]. To investigate the anti-atherosclerotic effects of eupatilin, we employed an aortic ring assay. Cell sprouting areas of the aortic rings increased as a result of PDGF-BB treatment, while the addition of eupatilin attenuated the effect (**Fig. 2A**). Eupatilin treatment (10, 20, or 40 µM) of PDGF-BB-induced aortic rings resulted in the significant inhibition of sprouting areas (18.9 %, 61.1 %, and 79.8 %, respectively) (**Fig. 2B**).

To elucidate the mechanisms responsible for the inhibitory effects of eupatilin, we assessed the outcome of eupatilin treatment on PDGF-BB-induced proliferation and migration of HASMCs, which represent critical steps in the development of atherosclerosis. Eupatilin treatment significantly attenuated PDGF-BB-induced HASMCs proliferation (**Fig. 3A**). To evaluate the effects of eupatilin on PDGF-BB-induced migration in HASMCs, we performed two types of migration assay. The wound healing migra-

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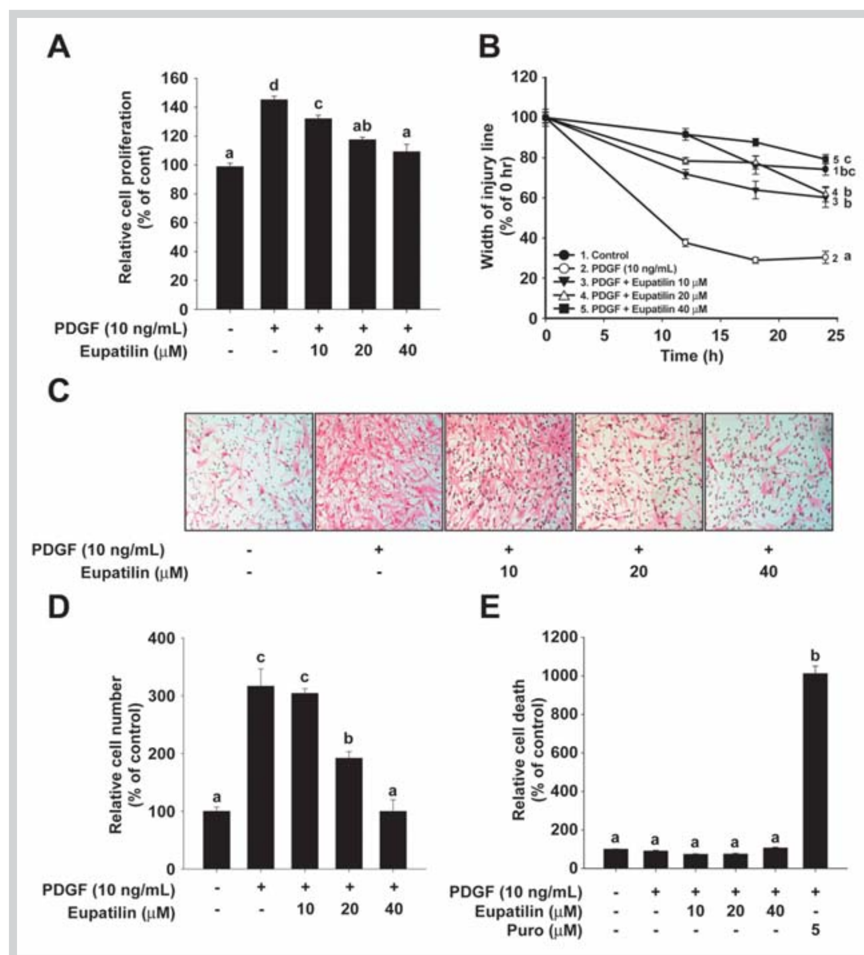

Fig. 3 Effects of eupatilin on PDGF-BB-induced proliferation and migration of HASMCs. **A** Inhibitory effect of eupatilin on PDGF-BB-induced proliferation of HASMCs. Serum-starved HASMCs were pretreated with 10, 20, or 40 µM eupatilin for 1 h, prior to stimulation with 10 ng/mL PDGF-BB for 48 h. Results were determined from three independent experiments. Data are represented as mean ± SEM values. **B–D** Inhibitory effects of eupatilin on PDGF-BB-induced migration in HASMCs. **B** Confluent HASMCs in serum-free medium were pretreated with 10, 20, or 40 µM eupatilin for 1 h before the addition of 10 ng/mL PDGF-BB. Thereafter, the widths of the injury lines were measured at 0, 12, 18, and 24 h. Results are expressed as the average widths of the injury lines relative to untreated controls at 0 h, as determined from three independent experiments. Data are represented as mean ± SEM values. **C** and **D** HASMCs seeded onto an inner chamber in serum-free medium were exposed to 10, 20, or 40 µM eupatilin and 10 ng/mL PDGF-BB for 8 h. **C** Representative images of migrated cells. **D** Migrated cells were counted as described in Materials and Methods. Results are expressed as % of the number of migrated cells relative to untreated controls as determined from three independent experiments. **E** Cytotoxicity test with eupatilin. Serum-starved HASMCs were pretreated with 10, 20, 40 µM eupatilin or 5 µM puromycin for 1 h before 10 ng/mL PDGF-BB was added for a further 48 h of treatment. Data are represented as mean ± SEM values of at least three independent experiments. Means with letters (a–c) within a graph are significantly different from each other at $p < 0.05$. (Color figure available online only.)

tion assay showed that eupatilin inhibited PDGF-BB-induced HASMC migration completely (▶ **Fig. 3B**). This finding was further supported by the results of the modified Boyden chamber assay, showing that eupatilin clearly inhibited PDGF-BB-induced HASMC migration (▶ **Fig. 3C**). Stimulation with PDGF-BB increased HASMC migration by approximately 317% compared to the untreated controls. The treatment of PDGF-BB-induced cells with eupatilin resulted in a dose-dependent decrease in the number of migrated cells (▶ **Fig. 3D**). In addition, eupatilin did not exhibit detectable cytotoxicity when treated with PDGF-BB at the effective dosages (▶ **Fig. 3E**).

The activation of PI3K and its downstream substrates Akt/p70S6K is crucial for PDGF-induced SMC migration and proliferation [27,28]. To elucidate the inhibitory mechanisms of eupatilin action on migration and proliferation, we next investigated its effect on phosphorylation of Akt and its downstream substrate p70S6K. Treatment with eupatilin significantly reduced PDGF-BB-induced phosphorylation of Akt at both Ser473 and Thr308, as well as phosphorylation of p70S6K to an extent lower than that achieved by LY294002 (a well-known PI3K inhibitor) at the same concentration (▶ **Fig. 4A–B**). To elucidate whether PI3K might be a molecular target of eupatilin for the inhibition of proliferation and migration of HASMCs, we next performed PI3K activity assays. Eupatilin inhibited PI3K activity with an effect slightly lower than LY294002 at the same concentration (▶ **Fig. 4C**). These results support the hypothesis that the effect of eupatilin on proliferation and migration in HASMCs is linked to the direct inhibition of PI3K activity.

Several studies have reported that stimulation by PDGF results in MAPK activation and that all three MAPKs play a role in the proliferation and migration of SMCs [19,27]. To further understand the inhibitory mechanisms of eupatilin, we next investigated its effects on MAPK signaling. Our results show that PDGF-BB-induced phosphorylation of p38-MAPK and MKK3/6 (which is the upstream regulator of p38-MAPK) were both significantly inhibited by eupatilin treatment with an effect relatively lower than that achieved by SB203580, a p38-MAPK pathway inhibitor (▶ **Fig. 5A–B**). Eupatilin exhibited a significant inhibitory effect on the phosphorylation of JNK, although its effect was inferior to that of SP600125 (a JNK pathway inhibitor) at the same concentration (▶ **Fig. 5C**). Eupatilin also significantly inhibited PDGF-BB-induced phosphorylation of MKK4 (an upstream regulator of JNK) with relatively lower inhibitory effects than AG1296, a PDGFR inhibitor, while SP600125 did not exhibit any detectable inhibitory effect (▶ **Fig. 5D**). Eupatilin treatment did not affect the phosphorylation of ERK in HASMCs (▶ **Fig. 5E**).

Since we observed that PI3K, MKK3/6, and MKK4 pathways can be targets of eupatilin for the inhibition of PDGF-induced proliferation and migration in HASMCs, we compared the effects of eupatilin on PDGF-BB-induced proliferation and migration of HASMCs with those of commercial PI3K, p38-MAPK, and JNK pathway inhibitors (LY294002, SB203580, and SP600125). Eupatilin exhibited equivalent or relatively stronger inhibitory effects on PDGF-BB-induced migration (▶ **Fig. 6A–B**) and proliferation (▶ **Fig. 6C**) of HASMCs than the commercial inhibitors without

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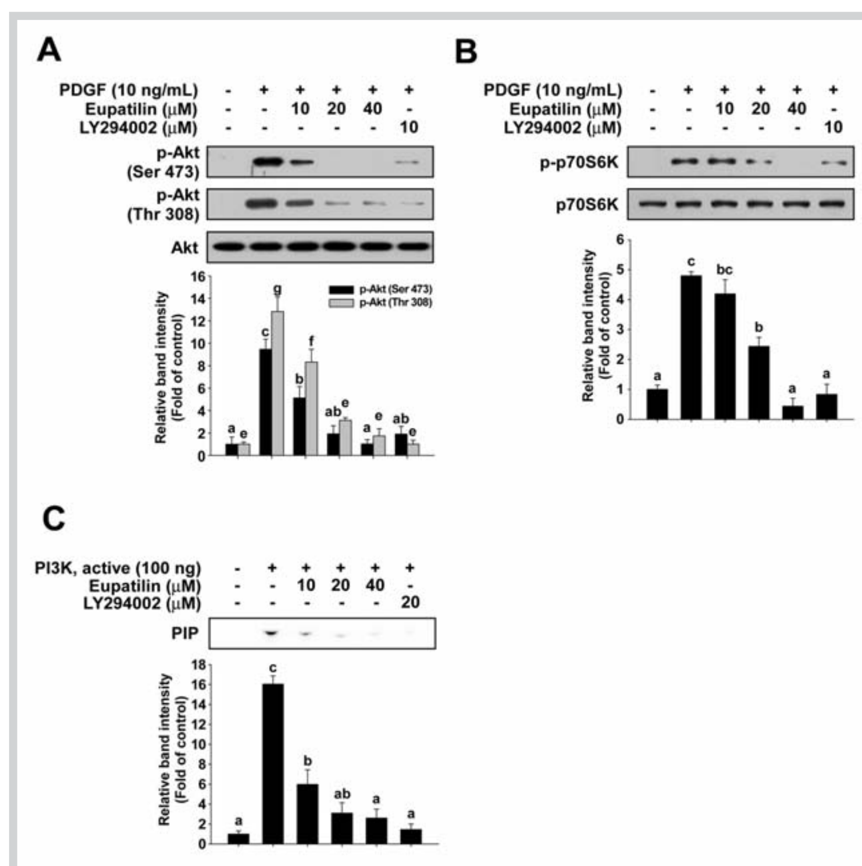

Fig. 4 Effects of eupatilin on PDGF-BB-induced phosphorylation of Akt and p70S6K in HASMCs and PI3K activity. **A** and **B** After pretreating the serum-starved HASMCs with 10, 20, 40 µM eupatilin or 10 µM LY294002 for 1 h, the cells were stimulated with 10 ng/mL PDGF-BB for 15 min. The levels of phosphorylated and total forms of Akt and p70S6K proteins were determined by Western blot analysis as described in Materials and Methods. Quantification of the phosphorylated proteins was normalized with total protein. Data are represented as mean ± SEM values of three independent experiments. **C** *In vitro* PI3K assay. Experiments were performed using 10, 20, or 40 µM eupatilin, or 20 µM LY294002 as described in Materials and Methods. Data are represented as mean ± SEM values of three independent experiments. Means with letters (a–c and e–g) within a graph are significantly different from each other at $p < 0.05$.

detectable cytotoxicity (**Fig. 6 D**) when treated alone at 40 µM dosages.

Discussion

SMCs are a major constituent of the media layer of blood vessels. Their proliferation and migration induce thickening of the aorta, which is a pivotal step in the development of atherosclerosis [29]. To better understand the effects of eupatilin on vascular thickening, we modified a rat aortic ring assay using matrigel, to determine the extent of cell sprouting mediated by proliferation and migration only within the media layer [19]. This method provided a clearer perspective of the specific inhibitory effects of eupatilin on PDGF-BB-induced aortic ring sprouting. We also observed that eupatilin exhibited anti-proliferative and anti-migratory effects on HASMCs within a similar concentration range.

A number of polyphenol compounds derived from plants have been reported to possess potent anti-inflammatory, anti-carcinogenic, and anti-cardiovascular properties. However, many of the cellular mechanisms responsible for these effects remain unclear. In recent years, it has been hypothesized that such inhibitory effects are due to a direct inhibition of signaling intermediates in cells [30]. Identifying the specific protein targets of phytochemicals is critical for a deeper understanding of the molecular mechanisms and the development of phytochemical-based preventive strategies for therapeutic settings [31].

PDGF-BB is a chemo-attractant for SMCs. It stimulates SMCs through the activation of multiple signaling cascades, including the PI3K/Akt and MAPK pathways [28, 32]. We found that eupatilin inhibited the phosphorylation of Akt, p38-MAPK, and JNK, but not ERK. This is in line with findings from our previous study showing that piceatannol inhibits PDGF-BB-induced HASMC migration through the direct inhibition of PI3K [11]. In the present study, eupatilin was also found to exert specific inhibitory effects on PI3K activity.

MKK3/6 is an upstream kinase of p38-MAPK and is involved in its activation by PDGF-BB. Its phosphorylation, as well as that of p38-MAPK, has been shown to be inhibited by red wine polyphenols [27]. Although MKK4 and 7 are direct upstream kinases of JNK, HASMCs lack MKK7 expression [33]. We found that eupatilin effectively inhibited the phosphorylation of MKK3/6 as well as of MKK4. These results suggest that the PI3K/Akt, MKK3/6/p38-MAPK, and MKK4/JNK pathways are all potential molecular targets of eupatilin for the inhibition of PDGF-induced proliferation and migration in SMCs. These multiple pathway targets of eupatilin may offer an explanation as to why eupatilin exhibited more potent inhibitory effects than the commercial single pathway inhibitors, although the inhibitory effects of eupatilin on each individual signaling pathway was relatively weaker than those of the commercial inhibitors alone. We also found that combination treatment with the three inhibitors exerted stronger inhibitory effects than single treatment (**Fig. 1S**, Supporting Information). Previous reports have shown that multi-pathway inhibition by combination treatment with such inhibitors exerts more potent inhibitory effects [34–37].

Numerous studies have revealed evidence for the beneficial effects of eupatilin on cellular mechanisms relevant to human health. The inhibition of the MAPK pathway is a likely mechanism responsible for some proportion of eupatilin's effects, while the compound has also exhibited anti-proliferative influences in ras-transformed human mammary epithelial cells, involving ERK

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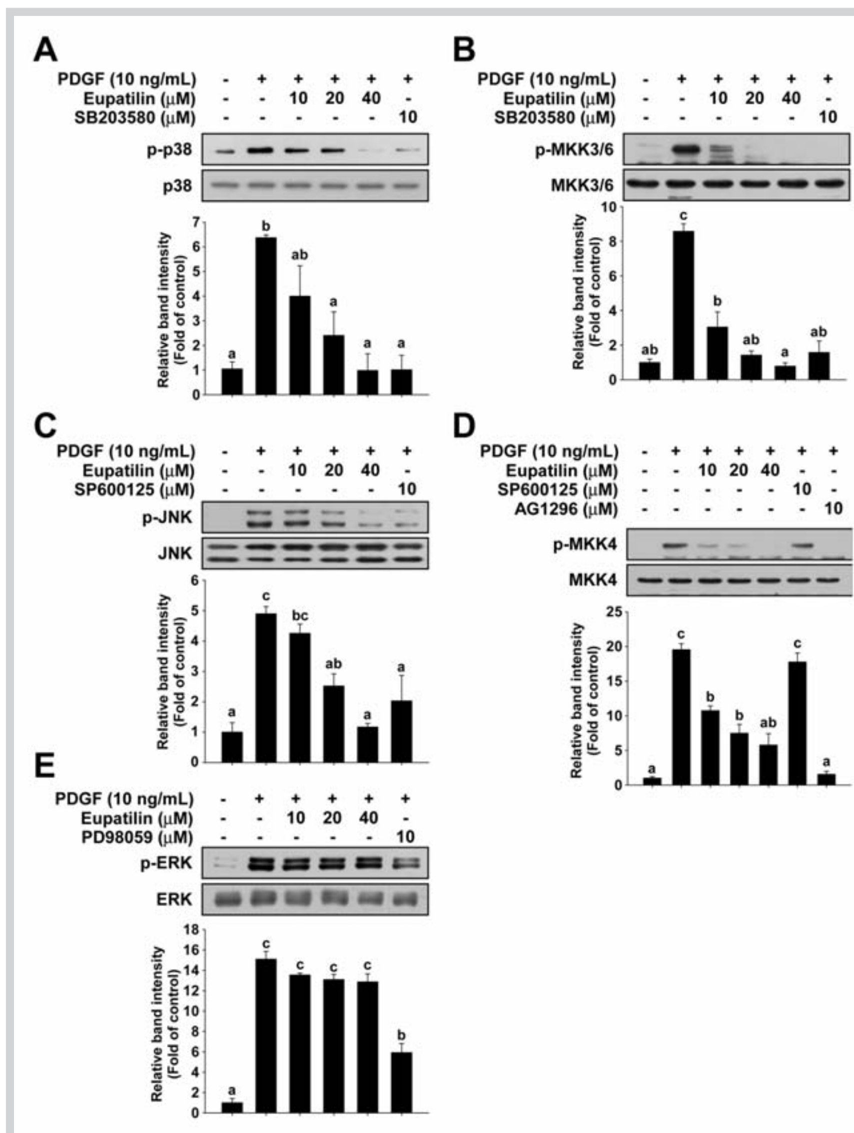

Fig. 5 Effects of eupatilin on phosphorylation of MAPKs pathways in HASMCs. After pretreating the serum-starved HASMCs with 10, 20, 40 µM eupatilin, 10 µM of SB203580 (**A** and **B**), SP600125, AG1296 (**C** and **D**), or PD98059 (**E**) for 1 h, the cells were stimulated with 10 ng/mL PDGF-BB for 5 min. The levels of phosphorylated and total MAPKs, MKK3/6, and MKK4 proteins were determined by Western blot analysis as described in Materials and Methods. Quantification of phosphorylated proteins was normalized with total protein. Data are represented as mean ± SEM values of three independent experiments. Means with letters (a–c) within a graph are significantly different from each other at $p < 0.05$.

inhibition. Additionally, eupatilin has been shown to abrogate H_2O_2-induced apoptosis in human gastric epithelial cells through the inhibition of ERK and JNK [38,39]. Interestingly, one report found evidence revealing an increased phosphorylation of ERK in eupatilin-treated ileal smooth muscle cells [40]. This is in contrast to our findings, which show that eupatilin does not inhibit PDGF-BB-induced ERK phosphorylation. A likely interpretation for this potential discrepancy is that eupatilin's ability to influence signaling homeostasis for anti-tumor, anti-apoptotic, or anti-atherosclerotic effects is dependent upon cell type, and many of these pathways are involved in diverse mechanisms. In addition, single polyphenols have been found to bind and inhibit a plethora of signaling molecules [41]. It is quite possible that eupatilin has additional unidentified targets that contribute to its effects on cell abnormality.

In summary, our results demonstrate that eupatilin inhibits PDGF-BB-induced rat aortic ring sprouting as well as the proliferation and migration of HASMCs. We report that this occurs concurrently with the inhibition of PI3K kinase activity and subsequent Akt phosphorylation. We also found that eupatilin attenuated MKK3/6 and MKK4 phosphorylation and the downstream kinases p38-MAPK and JNK (▶ Fig. 7). Taken together, these results suggest that eupatilin has the potential for further clinical investigation into its preventive effects against atherosclerosis.

Materials and Methods

Reagents

Purified eupatilin (purity ≥ 95%) dissolved in DMSO was kindly provided by Prof. Nam-In Baek of Kyung Hee University (Seoul, Korea) [26]. LY294002 (purity ≥ 98%), SB203580 (purity ≥ 98%), and SP600125 (purity > 98%) were acquired from CalBiochem and dissolved in DMSO. Stock solutions of all drugs were prepared in DMSO at 40 mM concentrations. For all experiments with eupatilin and/or inhibitors, PDGF-only (without drug) groups were incubated with 0.1% DMSO (representing the maximum concentration of DMSO for the highest drug dosage) as vehicle control, respectively. The antibody against *β*-actin was purchased from Sigma-Aldrich. Human PDGF-BB was supplied by R&D Systems. MCDB131 medium, streptomycin/penicillin, insulin, fetal bovine serum (FBS), and L-glutamine were purchased from Gibco-BRL. Epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and Sytox green dye were obtained from

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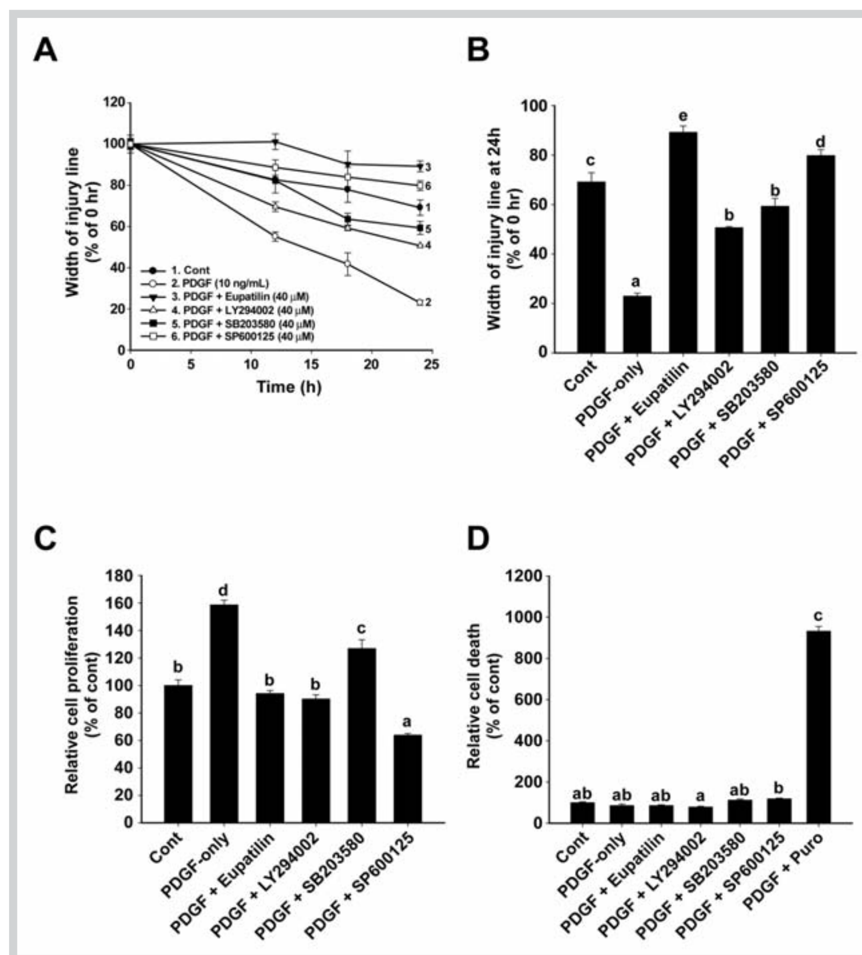

Fig. 6 Comparison of the effects of eupatilin, LY294002, SB203580, and SP600125 on PDGF-BB-induced migration and proliferation of HASMCs. **A** and **B** Inhibitory effects of eupatilin, LY294002, SB203580, and SP600125 on PDGF-BB-induced migration of HASMCs. Serum-starved HASMCs were pretreated with 40 µM samples for 1 h, prior to stimulation with 10 ng/mL PDGF-BB. Thereafter, the widths of the injury lines were measured at 0, 12, 18, and 24 h. Results are expressed as the average widths of the injury lines relative to untreated controls at 0 h, as determined from three independent experiments. Data are represented as mean ± SEM values. **C** Inhibitory effects of eupatilin, LY294002, SB203580, and SP600125 on PDGF-BB-induced proliferation of HASMCs. Serum-starved HASMCs were pretreated with 40 µM samples for 1 h, prior to stimulation of 10 ng/mL PDGF-BB for 48 h. Data are represented as mean ± SEM values of at least three independent experiments. **D** Cytotoxicity test with eupatilin, LY294002, SB203580, and SP600125. Serum-starved HASMCs were pre-treated with 40 µM LY294002, SB203580, SP600125, or 5 µM puromycin before 10 ng/mL PDGF-BB was added for a further 48 h of treatment. Data are represented as mean ± SEM values of at least three independent experiments. Means with letters (a–e) within a graph are significantly different from each other at $p < 0.05$.

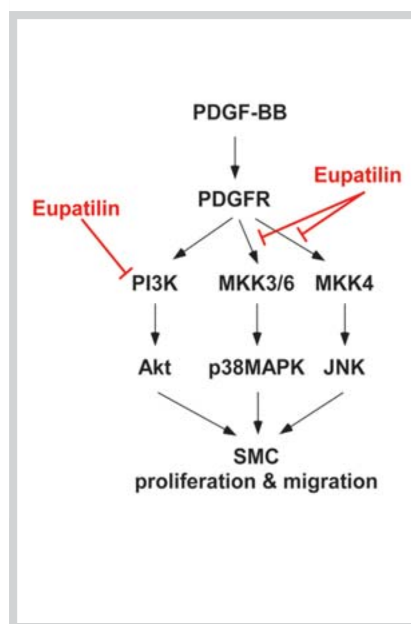

Fig. 7 Proposed model for the inhibitory effects of eupatilin on proliferation and migration in SMCs. PDGF-BB induces proliferation and migration of SMCs, via activation of the PI3K/Akt/p70S6K and MAPK pathways, subsequently leading to atherosclerotic development resulting from enhanced SMC motility. Eupatilin attenuates this proliferation and migration through the direct inhibition of PI3K activity, as well as phosphorylation of MKK3/6-p38 MAPK and MKK4-JNK. (Color figure available online only.)

Invitrogen. Antibodies against phosphorylated ERK (Tyr202/Tyr204), phosphorylated Akt (Ser473), total ERK and total JNK, and AG1296 were purchased from Santa Cruz Biotechnology. Antibodies against phosphorylated MKK3/6, phosphorylated MKK4, phosphorylated Akt (Thr308), phosphorylated JNK1/2, phosphorylated p38-MAPK, phosphorylated p70S6K, total MKK3/6, total MKK4, total p38-MAPK, total Akt, and total p70S6K antibodies were purchased from Cell Signaling Biotechnology. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) was purchased from USB Corporation. The active p110α subunit of the PI3K protein was obtained from Upstate Biotechnology. [γ-^{32}P]ATP was purchased from the Institute of Isotopes Co, Ltd (Budapest, Hungary), and the protein assay kit was obtained from Bio-Rad Laboratories.

Aortic sprouting assay

The experimental protocol was approved by the Animal Care and Use Committee of Seoul National University. The aortic sprouting assay was employed as previously described [19,42]. Briefly, the endothelium and adventitia of aortas from Sprague-Dawley rats (8 weeks old) were removed by enzymatic digestion, and the media layer of the aortas were cut into rings (1 mm wide). The rings were embedded in 48-well plates coated with Matrigel (BD Bioscience) and simultaneously treated with 10 ng/mL PDGF-BB and eupatilin, as indicated, in FBS-free medium. The rings were stained with methylene blue and photographed on day 5. Sprouting areas were quantitatively analyzed using Image J software.

Cell culture

HASMCs were isolated from normal human tissue with informed consent in accordance with the principles outlined in the Declaration of Helsinki, and supplied by Lonza. Cells were cultured in monolayers at 37 °C in a 5% CO_2 atmosphere in MCDB131 con-

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taining 10 % FBS, 2 mM L-glutamine, EGF, bFGF, insulin, and streptomycin/penicillin. All experiments were performed with HASMCs from passages 7 to 13.

Cell proliferation assay

HASMCs seeded in 24-well plates were serum-starved overnight and treated with indicated concentrations of eupatilin, LY294002, SB203580, or SP600125 for 1 h before 10 ng/mL PDGF-BB was added for the proliferation assay for 48 h. Next, 50 µL of MTT solution was added to each well containing 450 µL of medium and incubated for another 4 h at 37 °C. The medium was then removed, and 400 µL of dimethyl sulfoxide (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 proliferation were determined by reading the absorbance at 570 nm.

Wound healing migration assay

Cell migration was assessed using the wound healing migration assay as previously described [11]. Briefly, HASMCs (5×10^5) were seeded in a 6-well plate. After confluence, serum-starved cells were treated with 75 µM mitomycin C for 30 min to suppress cell proliferation. The cells were subjected to injury using a 2-mm wide tip before treatment with 10 ng/mL PDGF-BB alone or together with indicated concentration of eupatilin, LY294002, SB203580, or SP600125. The cells were allowed to migrate, and photographs were taken using an inverted microscope (× 200 magnification) and quantified using Image J software.

Boyden chamber migration assay

The Boyden chamber invasion assay was performed as previously described [11] using the Transwell system (Corning Costar). The lower side of the filter was coated with type I collagen (Collaborative Research), and the lower compartment was filled with MCDB131 medium containing 0.1 % bovine serum albumin and eupatilin treatment. Cultured cells (3×10^4) were resuspended in MCDB131 medium, placed in the upper compartment of the Transwell plate and allowed to migrate for 8 h. HASMCs were fixed with methanol and stained with haematoxylin and eosin. HASMCs on the upper surface of the filter were 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.

Assessment of cytotoxicity

Cytotoxicity was assessed with Sytox green, a membrane-impermeable DNA dye that enters dead cells as a result of altered membrane permeability and intercalates into the nucleic acid [43]. DNA-bound Sytox green can be detected at an excitation wavelength of 485 nm and an emission wavelength of 538 nm using a fluorescence microplate reader. Serum-starved cells grown in 96-well plates were treated with the indicated concentrations of eupatilin, LY294002, SB203580, SP600125, or 5 µM of puromycin as a positive control for 1 h, before 10 ng/mL PDGF-BB was administered for the cell death assay (48 h). Next, 1 µM Sytox green was added for 10 min before the number of dead cells was determined by fluorescence intensity.

Western blotting

Confluent serum-starved HASMCs were pretreated with indicated concentrations of eupatilin, LY294002, SB203580, SP600125, or AG1296 as positive controls for 1 h before being exposed to 10 ng/mL of PDGF-BB for different durations. Total cell lysates were prepared and subjected to Western blot as described previously [11]. Image J software was used for densitometry data analysis.

In vitro kinase assay

The *in vitro* kinase assays were performed according to the manufacturer's instructions. The active p110*α* subunit of the PI3K protein (100 ng) was incubated with eupatilin or LY294002 at 30 °C. The reactions were then incubated with 20 µL of 0.5 mg/mL phosphatidylinositol (Avanti Polar Lipids) at room temperature, followed by incubation with reaction buffer 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), which was previously activated by heating at 110 °C for 1 h. The resulting ^{32}P-labelled PI_3P was separated by thin layer chromatography, and the radiolabelled spots were visualized by autoradiography.

Statistical analysis

Where applicable, data are expressed as mean ± SEM values, and one-way analysis of variance (ANOVA) was used for comparisons in the experiments with multiple samples treatment. When ANOVA indicated statistical significance, Tukey's honestly significant difference (HSD) 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.

Supporting information

Data on the effects of pathway inhibitor combination treatment on PDGF-BB-induced proliferation and migration of HASMCs are available as Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

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