Pterostilbene Inhibits Vascular Smooth Muscle Cells Migration and Matrix Metalloproteinase-2 through Modulation of MAPK Pathway

Hsing-Chun Lin, Ming-Ju Hsieh, Chiung-Huei Peng, Shun-Fa Yang, and Chien-Ning Huang

Abstract: Smooth muscle cells (SMCs) migration and matrix metalloproteinase-2 (MMP-2) activation are main roles in atherosclerosis. Pterostilbene (trans-3, 5-dimethoxy-4-hydroxystilbene) is known to have various pharmacologic effects such as anti-inflammatory and anticarcinogenic properties. The present study aimed to investigate the anti-atherosclerotic property of pterostilbene in the rat smooth muscle cell (SMC) A7r9 cell lines and the underlying mechanisms. In this study, pterostilbene treatment significantly inhibited migration/invasion capacities of in A7r9 cell. Pterostilbene was also found to significantly decreased MMP-2 activity and expression by gelatin zymography and western blot assay in SMC. In the MAPK signaling pathway, western blot assay also indicated that pterostilbene up-regulated the phosphorylation of extracellular-signal-regulated kinase (Erk)1/2. Moreover, inhibition of Erk1/2 by specific inhibitors significantly abolished the pterostilbene-decreased expression of MMP-2 and migration/invasion capacities. These findings suggest that pterostilbene inhibited SMC migration and that MMP-2 activation could be mediated via Erk1/2 phosphorylation. It is further possible that pterostilbene could play a novel role in the treatment of atherosclerosis.

Keywords: matrix metalloproteinase-2, migration, pterostilbene, smooth muscle cells

Practical Application: Pterostilbene is a plant polyphenol compound that is principally found in blueberries. In this study, we found that pterostilbene could inhibit SMCs migration via down-regulation of MMP-2. Particularly, expression of MMP-2 was found to be strongly associated with the phosphorylation of Erk1/2.

Introduction

In the United States and other Western countries, cardiovascular disease (CVD) is the major cause of illness and death. Diabetes, hypertension and dyslipidemia are risk factors that contribute to the increased incidence of cardiovascular disease. Atherosclerosis is a progressive pathological disorder leading to cardiovascular and cerebrovascular diseases, including endothelial cell dysfunction, vascular smooth muscle cell (VSMC) proliferation and migration, fibrous cap formation, plaque rupture and thrombosis. Particularly, SMC proliferation and migration has been identified as the main step in the progression of vascular diseases, such as atherosclerosis and restenosis (Ross 1990; Yu and others 2008). Due to extracellular matrix (ECM) damage or remodeling contributes to the formation, progression, and clinical expressions of rheumatoid arthritis (Weber and De Bandt 2000), malignant tumors (Ohtani 1998), aortic aneurysm (MacSweeney and others 1994), and atherosclerosis (Libby 1995, 2000). Consequently, migration of VSMCs from the tunica media to the subendothelial even more requires proteinases which degrade ECM and prevent its remodeling.

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Matrix metalloproteinases (MMPs) are a family of Zn^{2+}-dependent endopeptidases capable of cleaving components of ECM (Falk 1999; Creemers and others 2001). The production of gelatinases (MMP-9 and MMP-2) and SMCs migration may play especially key roles in the pathogenesis of neointima formation and atherosclerosis (Bäck and others 2010). According to Bäck's reports, MMP-2 is mainly expressed in the normal vascular smooth muscle cells, and MMP-9 is expressed in leukocytes (Bäck and others 2010). Moreover, the activity of MMPs is controlled by specific inhibitors known as tissue inhibitors of MMPs (TIMPs). The balance between production of MMPs and TIMPs is critical for the maintenance of the homeostasis of the ECM (Birkedal-Hansen and others 1993; Strongin and others 1993; Jezierska and Motyl 2009).

Pterostilbene (trans-3, 5-dimethoxy-4-hydroxystilbene), a flavonoid extensively found in blueberries and grapes, confers potent anti-inflammatory and anticarcinogenic properties (Remsberg and others 2008; Wu and others, 2015). The chemical structure of pterostilbene is similar to resveratrol, but is significantly more bioavailable on its anticancer properties and antioxidant capacity (McCormack and others 2012; Weng and Yen 2012). Moreover, in vivo and in vitro studies have demonstrated pterostilbene has anticarcinogenic effects to inhibit cell migration and induce cell apoptosis in breast, colon, and melanoma cancers (McCormack and others 2012). Even previous research also indicated that pterostilbene inhibited SMC proliferation via down-regulating the phosphorylation of Akt (Park and others 2010). However, it is unknown whether Mitogen-activated protein kinases (MAPK) and Phosphatidylinositol 3 kinases (PI3K) pathways were involved in SMC migration. Expression of MMPs was found to be strongly associated with the MAPK pathway, including extracellular

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regulated kinase 1/2 (ERK1/2), c-Jun NH2-terminal kinase (JNK), and p38 (Stoclet and others 2004; Chien and others 2013). In this study, we thus investigated that the effect of pterostilbene on SMCs migration and MMP-2 activation mechanism.

Materials and Methods

Cell culture

Rat smooth muscle cell line (A7r5) were maintained in low glucose DMEM culture medium containing 10% heat-inactivated FBS, 2 mmol/L L-glutamine, 1.5 g/L sodium bicarbonate, and 10 mmol/L HEPES. All experiments were treated in high glucose DMEM, and in different concentrations (0, 10, 20, and 40 μM) of pterostilbene. The structure of pterostilbene is shown in Figure 1A.

Cell viability assay (MTT assay)

The survival activity of pterostilbene on A7r9 was investigated using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay (Yu and Lin 2010). A7r9 cells were seeded in 24-well culture plates at a density of 2×10^4 cells per well in culture medium and added with various concentrations of pterostilbene for 24 and 48 h. After remove medium, MTT reagent (final concentration 0.5 mg/mL) was added for 4 h. Then, the solution absorbance was directly measured by absorbance intensity at OD 565 nm.

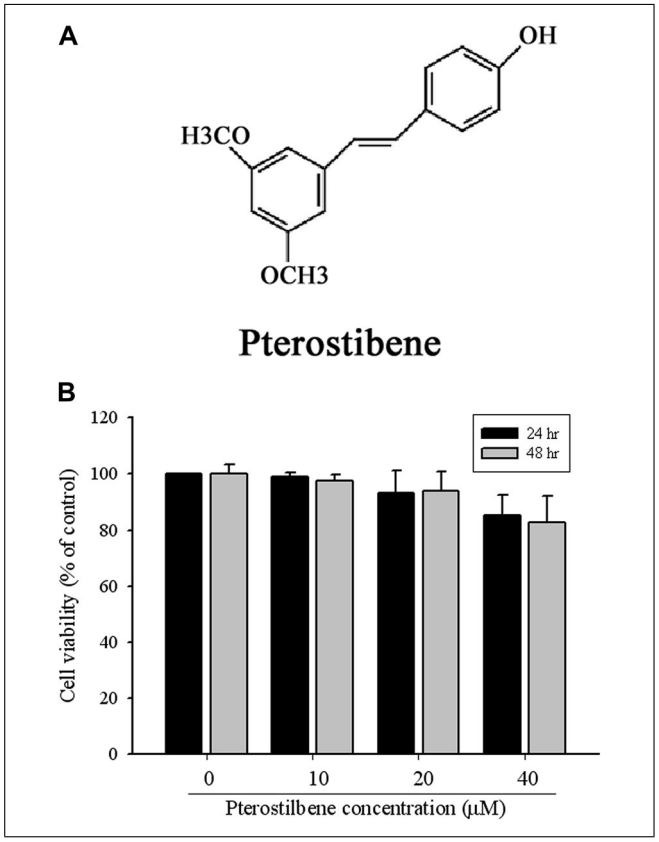

Figure 1–Effects of pterostilbene on cell viability. (A) Structure of pterostilbene. (B) A7r5 cells were treated with various concentrations (0, 10, 20, and 40 μM) of pterostilbene for 24 and 48 h. Cell viability was determined using an MTT assay. The values represented the means ± SD of at least 3 independent experiments.

Invasion assay/migration assay

Invasion and migration assay of A7r5 cells were determined by 48 well Boyden chamber (Neuro Probe, Cabin John, Md., U.S.A.), and the invasion assay was coated with Matrigel (Collaborative Biomedical Products, Bedford, Ma., U.S.A.) on the upper components of the Boyden chamber (Yang and others 2013). Cells were treated with different concentrations (0, 10, 20, and 40 μM) of pterostilbene for 24 h, and added to the upper components of the Boyden chamber (1×10^4 cell/well). The conditioned medium (10% FBS, DMEM) was added to the lower compartments of the Boyden chamber. After incubation for 24 h, the cells on the upper side filter were removed by cotton, and then fixed with methanol for 10 min. Finally, air-dried for 5 min in Giemsa (1:20) staining 1 h and randomly selected 3 horizons per well under the microscope at 400×.

Wound healing assay

A7r9 cells were seeded in a 6-well plate for 16 h (8×10^4 /well). After scratch the wound by using a pipette tip, the sample was incubated with DMEM medium containing 0.5% FBS and treated with different concentrations (0, 10, 20, and 40 μM) of pterostilbene for 0, 12, and 24 h. Imaging was performed on a phase-contrast microscope (model CK40, Olympus) as described previously (Huang and others 2009).

Gelatin zymography

MMP-2 activity in serum-free DMEM medium of maintained A7r5 cells was detected by SDS-PAGE (10 %) containing 0.1% gelatin. Equal volumes of samples were mixed with loading buffer, which loaded onto the gel and electrophoretic separation. Then, the gels were washed in wash buffer (50 mmol/L Tris-HCl, 5 mmol/L $CaCl_2$, 0.02 % NaN_3, and 2.5% Triton X-100) and incubated for 18 h at 37 °C with reaction buffer (50 mmol/L Tris-HCl, 5 mmol/L $CaCl_2$, and 0.02% NaN_3). After the reaction, the gels were stained 30 min with stained buffer (Coomasssie Brillant Blue R-2500) and destained in 5% methanol and 7% acetic acid. Gelatinolytic activity was identified as clear bands on a blue background.

Western blot analysis

Cellular proteins were extracted into lysis buffer containing 150 mM NaCl, 1% (v/v) Triton X-100, and a cocktail of protease inhibitors. The same amount of protein (20 μg) was subjected to 10% SDS-PAGE, and blotted on the PVDF membrane. After blocking with PBS-Tween 20 (PBST) buffer containing 50 g/L nonfat milk, the membranes were incubated with primary antibody at 4 °C overnight, and then with secondary peroxidase-conjugated anti-rabbit IgG (Vector). The blots were washed and then developed with an enhanced chemiluminescence kit (PerkinElmer Life Science, Boston, Ma., U.S.A.).

Statistical analysis

All results were presented as mean ± SD. Statistical differences were performed using the ANOVA test. A value of $P < 0.05$ was taken to be statistically significant.

Results and Discussion

Migration and MMPs activation of VSMCs are important stages in the development of atherosclerosis and restenosis (Jones and others 2003). In recent years, mounting evidence has demonstrated that polyphenols are found in vegetables, fruits, cereal, herbs, and

so on (Gharras 2009). Due to polyphenols have powerful benefits in cardiovascular diseases and cancers (Petti and Scully 2009). Pterostilbene is one form of plant polyphenol compound that is principally found in blueberries, with approximately 15 μg of pterostilbene deriving from 100 g of blueberries (Rimando and others 2004). In human study, the safety dosage of pterostilbene can be up to 250 mg/d for 6 to 8 wk (Riche and others 2013). This compound has multiple pharmacologic effects, including being an antioxidant, anti-inflammatory (Remsberg and others 2008), antidiabetic (Pari and Amarnath Satheesh 2006), anti-atherogenic and anticancer (Stoclet and others 2004). Especially in atherosclerosis, pterostilbene has been found to effectively inhibit SMC proliferation and endothelial cell apoptosis in in vitro studies (Park and others 2010; Zhang and others 2013). Moreover, pterostilbene has advantages over resveratrol, including higher oral absorption, higher bioavailability and longer half-life (Remsberg and others 2008; Lin and others 2009). The cytotoxity of pterostilbene on A7r5 cells was detected using MTT assay. A7r5 cells were incubated for 24 and 48 h in a 24-well plate with various concentrations of pterostilbene (0, 10, 20, and 40 μM). The results of the incubation with various concentrations of pterostilbene are shown in Figure 1B. Figure 1B illustrates that pterostilbene was not cytotoxic to the A7r5 cells. The range of concentrations was explored in subsequent experiments.

VSMC migration and matrix remodeling both require the action of proteinases, among which MMPs and TIMPs may play key roles (Woessner 1991). The effect of pterostilbene on MMP-2 activation was detected using gelatin zymography. A7r5 cells were pretreated with various concentrations of pterostilbene (0, 10, 20, and 40 μM) for 24 h. Then, the supernatants of A7r5 cells were used to test the inhibitory effect of pterostilbene on MMP activity. As shown in Figure 2A, MMP-2 secretion was suppressed by pterostilbene in a dose-dependent manner. The effects of pterostilbene on MMP-2 and TIMP-2 expression were determined by Western blotting analysis. A7r5 cells were pretreated with various concentrations of pterostilbene (0, 10, 20, and 40 μM) for 24 h. The results, illustrated in Figure 2B, were that the inhibitory effect of pterostilbene on MMP-2 expression. Although gelatinases of MMPs have similar substrate specificities, there are differences

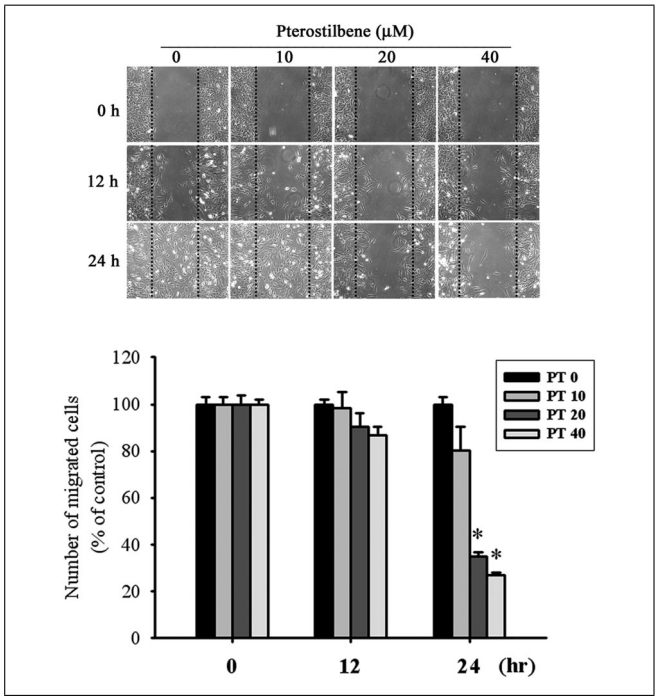

Figure 3–Effect of Pterostilbene on in vitro wound closure in A7r5 cells. A7r5 cells were treated with various concentrations of pterostilbene at 0, 12, and 24 h (photograph magnification, 400×). In the different time points, phase-contrast pictures of the wounds at 3 different locations were taken. A quantitative assessment of the mean number of cells in the denuded zone was the mean ± SD ($n = 3$). *$P < 0.05$ was considered statistically significant.

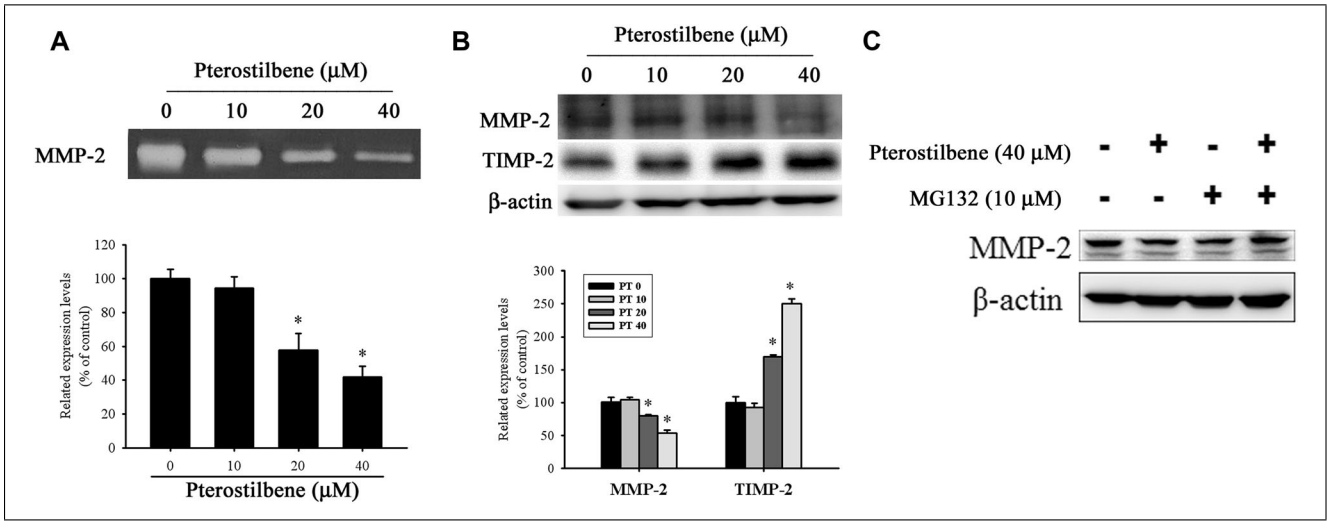

Figure 2–Effect of pterostilbene on the matrix metalloproteinase-2 (MMP-2) activity and expression in A7r5 cells (A). A7r5 cells were pretreated with different concentration pterostilbene (0, 10, 20, and 40 μM) for 24 h. The activity of MMP-2 was assessed by gelatin zymography. The band intensities were quantified with image analysis system software. Values represent mean ± SD, $n = 3$. *$P < 0.05$ was considered statistically significant. (B) Analysis of the MMP-2 and TIMP-2 proteins expressed in A7r5 cells by pterostilbene using Western blot analysis. A7r5 cells were pretreated with different concentration pterostilbene for 24 h. The expression of MMP-2 and TIMP-2 was assessed by western blot analysis. Representative western blot showing MMP-2 and TIMP-2 protein levels in cell lysates and β-actin (bottom). (C) A7r5 cells were pre-treated with MG132 (10 μM) for 1 h, and then incubated in the presence or absence of Pterostilbene (40 μM) for 24 h and cells were used for western blotting as described in the Materials and Methods section. The band intensities were quantified with image analysis system software. Values represent mean ± SD, $n = 3$. *$P < 0.05$ was considered statistically significant.

in regulating their expression. The expression of MMP-2 (gelatinases A, 72 kDa) in particular, which is found in VSMCs, is not stimulated by either cytokines or growth factors (Fabunmi and others 1996; Yu and others 2008). In contrast, MMP-9 (gelatinases B, 92 kDa) responds to several inducer and cell types (Cho and others 2000; Cimmino and others 2013). Moreover, the present study's zymography data indicated that the secreted protein level of MMP-9 from A7r5 cells was quite low. Therefore, we conclude that MMP-2 is important proteases of SMC migration. The findings of gelatin zymography and western blot (Figure 2A and B) reveal that pterostilbene inhibited the activity and protein expression of MMP-2. In addition, pterostilbene induced TIMP-2 expression in A7r5 cells (100 ± 8.3%, 93.1 ± 6.1%, 169.6 ± 2.6%, and 250.4 ± 7.4%, respectively). Previous research reported that TIMP-1 is a specific inhibitor of MMP-9, but TIMP-2 is a specific inhibitor of MMP-2 (Strongin and others 1993; Jezierska and Motyl 2009). In our study, we also found that pterostilbene induced the protein expression of TIMP-2 (Figure 2B). However, pterostilbene did not decrease the mRNA expression of MMP-2, as indicated by RT-PCR and real-time PCR (data not shown). To detect the real effect of pterostilbene on MMP-2, we determine whether pterostilbene could accelerate degradation of MMP-2 rather than decrease the expression of this protein. As shown in Figure 2C, western blotting assay revealed that pterostilbene accelerated MMP-2 protein degradation, which was prevented by MG132, a potent proteasome inhibitor.

In 2008, Pan and colleagues proved that pterostilbene can suppress invasive and metastatic activities in the MCF-7 breast cancer cell model (Pan and others 2011). To examine the role of pterostilbene plays in the aggressive phenotype of A7r5 cells, we performed wound healing and Boyden chamber assays, evaluating the effect of pterostilbene produced on the motility, migration and invasion abilities of a7r5 cells. After pretreatment with various concentrations of pterostilbene, wound healing and migration/invasion assays revealed pterostilbene inhibitory activity on A7r5 cells mobility. As shown in Figure 3, the result of the wound healing assay shows that pterostilbene decreased A7r5 cells mobility. The effect of migration on A7r5 cells was also inhibited by pterostilbene in a dose-dependent manner (Figure 4A). Similarly, we observed that pterostilbene inhibits SMC invasion (Figure 4B). We also investigate the effect of pterostilbene on pro-atherosclerotic factor-induced cell migration. As shown in Figure 4C, treatment with oxidized-LDL (ox-LDL) substantially increased cell migration, whereas pterostilbene reduce ox-LDL induced A7r5 cells mobility.

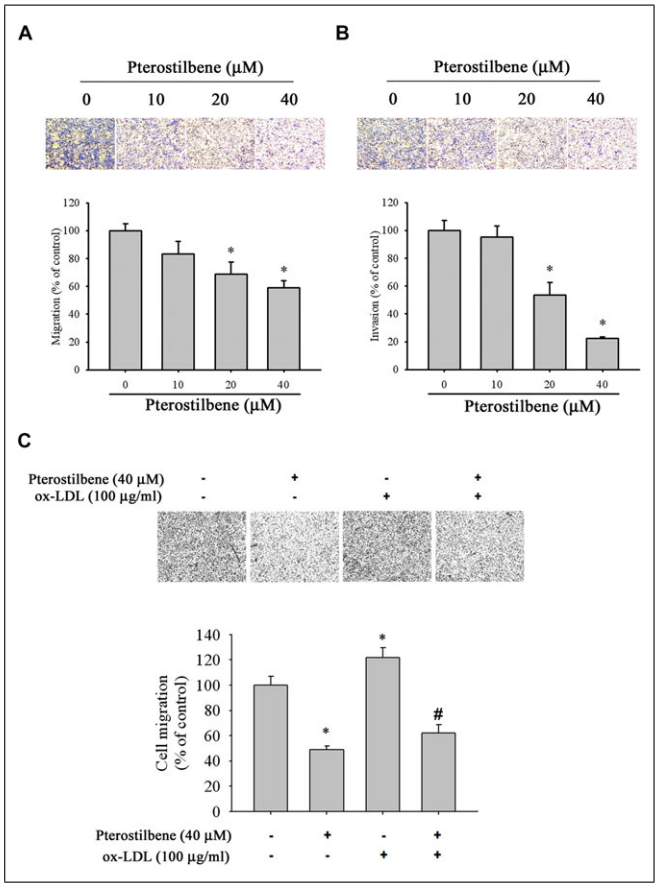

Figure 4–Effect of Pterostilbene on cell migration and cell invasion in A7r5 cells. (A) After being treated with Pterostilbene at a concentration of 0, 10, 20, and 40 μM for 24 h, the cell migration were measured using a Boyden chamber for 24 h. (B) After being treated with Pterostilbene at a concentration of 0, 10, 20, and 40 μM for 24 hs, the cell invasion were measured using a Matrigel-coated Boyden chamber for 24 hs. (C) After being treated with Pterostilbene at a concentration of 40 μM in the presence or absence of oxidized-LDL (ox-LDL) (100 μg/mL) for 24 h, the cell migration were measured using a Boyden chamber for 24 h. The values represented the means ± SD of at least 3 independent experiments. *P < 0.05 compared to the vehicle group. #P < 0.05 as compared with the Ox-LDL treated only.

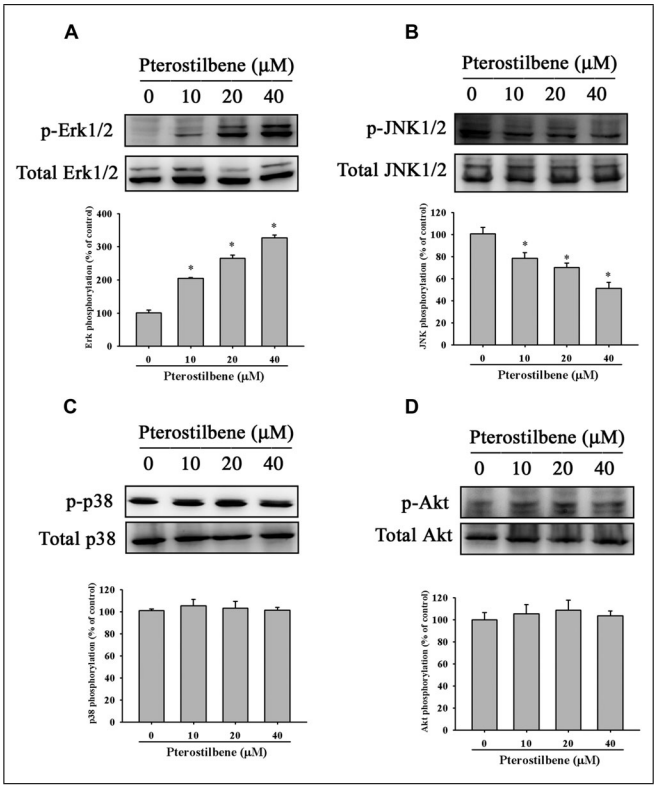

Figure 5–Effects of Pterostilbene on the MAPK and Akt signaling pathways in A7r5 cells. A7r5 cells were treated with various doses of pterostilbene (0, 10, 20, and 40 μM) for 24 h and whole cell lysates prepared from these cells were used for western blot analysis with (A) anti-ERK1/2, (B) anti-JNK1/2, (C) anti-p38, and (D) anti-Akt (total and phosphorylated) antibodies as described in the Materials and Methods section. Values represent mean ± SD, $n = 3$. *P < 0.05 was considered statistically significant.

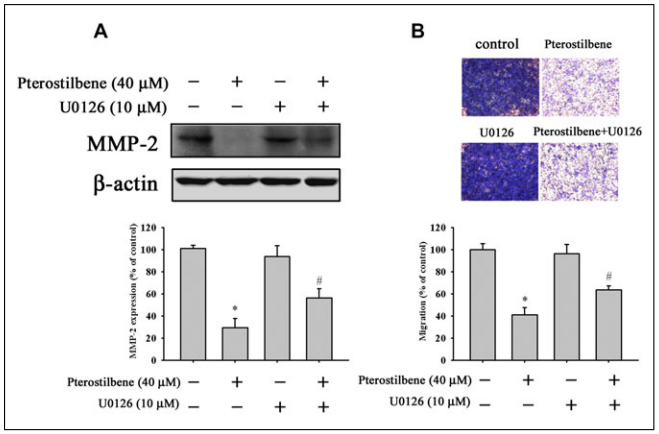

Figure 6–Effects of MEK inhibitor (U0126) and Pterostilbene on MMP-2 activity and migration of A7r5 cells. A7r5 cells were pretreated with U0126 (10 μM) for 1 h, and then incubated in the presence or absence of Pterostilbene (40 μM) for 24 h. (A) The culture media were used as subjects for analysis of MMP-2 activity, and cells were used for migration assay (B) as described in the Materials and Methods section. The values represented the means ± SD of at least 3 independent experiments. *P < 0.05 as compared with the control. #P < 0.05 as compared with the Pterostilbene treated only.

Previous studies have indicated that the activation of MAPK and PI3K signal transduction pathway can regulate MMP expression and SMC migration (Lin and others 2007; Ong and others 2011). The effect of pterostilbene on MAPK and Akt/PI3K signal pathway expression was detected. After treatment with various concentrations of pterostilbene (0, 10, 20, and 40 μM) for 24 h, pterostilbene was observed to increase the phosphorylation of Erk1/2 (Figure 5A) but reduce the phosphorylation of JNK1/2 on A7r5 cells (Figure 5B). However, the phosphorylation of the p38 and Akt remained unaffected (Figure 5C and D). To determine whether pterostilbene inhibited cell migration was caused mainly by up-regulating the phosphorylation of ERK1/2, we investigated its effects on specific inhibitors of ERK1/2 (U0126) in A7r5 cells. After the cells were pretreated with U0126 for 30 min, then treated with pterostilbene for 24 h, the MMP-2 expression and cell migration ability in this group was increased significantly in contrast with the pterostilbene only group (Figure 6A and B). These results indicated that pterostilbene inhibited MMP-2 expression and migration ability via Erk1/2 activation in A7r5 cells.

Conclusions

CVD due to atherosclerosis causes morbidity and mortality increase worldwide, and is strongly associated with inflammation. Pterostilbene is one form of plant polyphenol compound that is principally found in blueberries. In this study, pterostilbene was found to inhibit SMCs migration to prevent atherosclerosis, thereby decreasing MMP-2 expression.

Acknowledgments

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Author Contributions

Huang CN, Yang SF, Peng CH, and Hsieh MJ were responsible for the study concept and design. Lin HC mainly wrote the manuscript. All authors participated in the revise of the manuscript critically and approved the final version.

Conflict of Interest: None.

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