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Olibanum Extract Inhibits Vascular Smooth Muscle Cell Migration and Proliferation in Response to Platelet-Derived Growth Factor

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Olibanum (*Boswellia serrata*) has been shown to have anti-inflammatory, anti-arthritic and anti-cancer effects. This study determined the role of a water extract of olibanum in platelet-derived growth factor (PDGF)-stimulated proliferation and migration of rat aortic smooth muscle cells (RASMCs). PDGF-BB induced the migration and proliferation of RASMCs that were inhibited by olibanum extract in a dose-dependent manner. The PDGF-BB-increased phosphorylation of p38 mitogen-activated protein kinase (MAPK); the heat shock protein (Hsp) 27 was significantly inhibited by the olibanum extract. The effects of PDGF-BB-induced extracellular signal-regulated kinase1/2 was not altered by the olibanum extract. Treatment with olibanum extract inhibited PDGF-BB-stimulated sprout out growth of aortic rings. These results suggest that the water extract of olibanum inhibits PDGF-BB-stimulated migration and proliferation in RASMCs as well as sprout out growth, which may be mediated by the inhibition of the p38 MAPK and Hsp27 pathways.

Key Words: Olibanum, Migration, Proliferation, Rat aortic smooth muscle cells, Mitogen-activated protein kinase

INTRODUCTION

Olibanum is a resin obtained from *Boswellia serrata*, an Indian herb used as incense. This resin has been shown to have anti-inflammatory and -arthritic effects and has been extensively used in oriental medicine for the prevention and/or treatment of inflammatory disorders, including arthritis, asthma and inflammatory bowel disease (Gupta et al., 1998; Kimmatkar et al., 2003). The resin extracts have been shown to have anti-cancer effects in leukemia and brain tumors (Winking et al., 2000). The extracts are also known to influence cellular responses such as the proliferation of liver cancer cells and lymphocytes (Gayathri et al., 2007), and the migration of leukocytes (Sharma et al., 1988). However, it is not been known whether olibanum extracts affect vascular smooth muscle cell behavior, such as migration and proliferation.

Many vascular diseases are characterized by the abnormal accumulation of vascular smooth muscle cells in the intima of vessels, a process that is thought to occur, in part, as a result of the migration and proliferation of these cells from the tunica media (Ross, 1999; Lee et al., 2009). Vascular smooth muscle cell migration and proliferation is induced by various inflammatory cytokines and growth factors. PDGF has been recognized as a major mitogen and one of the most important growth factors; it has been shown to stimulate vascular smooth muscle cell proliferation and migration (Grotendorst et al., 1981; Raines, 2004; Millette et al., 2006). PDGF is known to induce signaling molecules that are associated with the activation of mitogen-activated protein kinases (MAPKs) (Pearson et al., 2001). MAPKs belong to a family of serine/threonine-specific protein kinases, consisting of three isoforms: extracellular signal-regulated kinase (ERK) 1/2, p38 MAPK, and stress-activated protein kinase/c-*Jun* N-terminal kinase (JNK) (Geraldes et al., 2002; Kim et al., 2004). These MAPKs are involved in the regulation of the migration and proliferation of vascular smooth muscle cells (Liu et al., 2007). Most of the PDGF-stimulated signaling cascade requires the activation of MAPKs. The MAPK pathway mediates migration and proliferation in response to PDGF in the vascular smooth muscle (Lee et al., 2007). The small heat shock protein (Hsp) 27, a physiological substrate for p38 MAPK, modulates the polymerization of actin by its phosphorylation (Hedges et al., 1999). The Hsp27/p38 MAPK pathway plays an important role in actin cytoskeleton remodeling and smooth muscle cell migration (Hedges et al., 1999). Moreover, Hsp27 has been reported to be involved in the regulation of smooth muscle cell proliferation (Champagne et al., 1999; Salinthone et al., 2007).

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ABBREVIATIONS: PDGF, platelet-derived growth factor; RASMC, rat aortic smooth muscle cells; MAPK, mitogen-activated protein kinase; Hsp, heat shock protein; ERK, extracellular signal-regulated kinase; JNK, c-*Jun* N-terminal kinase; DMEM, dulbecco modified eagle medium; FBS, fetal bovine serum; OWE, olibanum water extract.

A variety of studies on herbal medicines have been performed for the prevention and cure of patients with vascular disorders, including hypertension and atherosclerosis. However, to date, the responses of proliferation and migration, in rat aortic smooth muscle cells (RASMCs), to olibanum extract, have not been studied. The present investigation was designed to determine the role of olibanum extract in the proliferation and migration response of RASMCs to PDGF using the water extraction method.

METHODS

Materials

PDGF-BB was purchased from R&D Systems (Minneapolis, MN, USA) and Matrigel was purchased from BD Bioscience (San Jose, CA, USA). Dulbecco modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT, USA). The antibodies used included anti-p38 MAPK, anti-phospho p38 MAPK (Cell signaling, Beverly, MA, USA), anti-phospho Hsp27 (Affinity BioReagents, Golden, CO, USA), anti-ERK1/2, anti-phospho ERK1/2 (Promega, Madison, WI, USA), and anti-β-actin (Sigma, St Louis, MO, USA).

Preparation of olibanum extract

The olibanum was pounded in a mortar. The powder (25 g) was mixed with 200 ml of distilled water and stirred overnight at room temperature. This mixture was centrifuged at 1,500 g for 10 min and the supernatant was collected. Thereafter, the supernatant was again centrifuged at 2,500 g for 10 min and successively at 10,000 g for 20 min, and then was filtered. The filtrates were stored at $-80^{\circ}C$ and then freeze-dried to yield 3.875 g of water soluble extract.

Cell preparation and culture

All experiments were conducted according to the institutional guidelines of Konkuk University, Republic of Korea. RASMCs were enzymatically isolated from male Sprague Dawley rats (5 weeks old, 160 g, n=2, Daehanbiolink, Chungju, Republic of Korea), and cultured in DMEM containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 200 mM glutamine. RASMCs, used at passages 5~8, were grown to 70~80% confluence and starved in FBS-free DMEM for 24 h.

Immunoblotting

The cells were treated with PDGF-BB or olibanum extract and were lysed with a cold buffer (20 mM HEPES [pH 7.5], 1% Nonidet P-40, 150 mM NaCl, 10% glycerol, 10 mM NaF, 1 mM Na_3VO_4, 2.5 mM 4-nitrophenylphosphate, 0.5 mM PMSF, and 1 tablet of complete proteinase inhibitor cocktail [Roche, Indianapolis, IN, USA]). The proteins (30~50 μg/lane) were separated on 8~12% polyacrylamide SDS gels, and then transferred electrophoretically to a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). The membranes were incubated overnight at $4^{\circ}C$ with antibodies diluted 1 : 1,000~2,000 and used for the secondary antibodies. The blots were then incubated with enhanced chemiluminescence reagents (Amersham Pharmacia, Piscataway, NJ, USA) and exposed to photographic film.

Migration assay

Cell migration was examined in 48-well Boyden microchemotaxis chambers (Neuro Probe, Cabin John, MD, USA). Polycarbonate membranes with 8 μm pores (Neuro Probe) were coated with 0.1 mg/ml type I collagen isolated from rat's tail tendon (BD Bioscience) and then dried for 60 min. The bottom chamber was loaded with 3×10^4 cells and the membrane was laid over the cells. The microchamber was then inverted and incubated at $37^{\circ}C$ for 120 min. The chamber was then returned to an upright position, and the upper wells were loaded with DMEM containing 0.1% BSA, PDGF-BB, and olibanum extract. The chamber was then incubated at $37^{\circ}C$ for 90 min, and the membranes were fixed and stained using Diff-Quik (Baxter Healthcare, Miami, FL, USA). The number of cells that had migrated through the membrane was determined by counting four randomly chosen regions of each well by microscopy (×400). All samples used in the experiments contained 0.1% dimethyl sulfoxide, which did not affect the RASMC migration.

Proliferation assay

RASMC proliferation was performed with a 5-bromo-2'-deoxyuridine (BrdU) incorporation assay (Roche). Cells, which were seeded in 96-well microtiter plates at a density of 2×10^3 cells/well for 12 h, were incubated in FBS-free DMEM for 6 h and then treated with olibanum extract and PDGF-BB for 36 h. The cells were treated with BrdU-labeling solution (10 μM) and then incubated for 12 h. After the denaturation of the DNA, peroxidase-labeled anti-BrdU monoclonal antibodies were added and the samples incubated at room temperature for 90 min. The BrdU-antibody complexes were detected with a Victor 3 luminometer (PerkinElmer, Boston, MA, USA).

Cell viability assay

The cell viability was measured by an 2,3-bis [2-methyloxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT) assay using the WelCount™ cell viability assay kit (WelGENE, Daegu, Korea). The RASMCs were incubated in 96-well plates (1×10^4 cells/well) for 24 h at $37^{\circ}C$ and treated with XTT (200 μg/ml) per each well. After the incubation of the plates in the presence of XTT dye, for 4 h to induce the formation of formazan dye, the samples were quantitated with an enzyme-linked immunosorbent assay reader at 450 nm.

Aortic ring assay

Ex vivo migration of the RASMCs was measured by an aortic ring assay using Matrigel, with some modifications of a previously reported method (Nicocia and Ottinetti, 1990). The endothelium and adventitium, of the aorta in Sprague Dawley rats (5 weeks, n=4), was removed enzymatically, the vessels were then cut into rings (1 mm). The rings were placed and embedded in 48-well plates coated with Matrigel (BD Bioscience), then PDGF-BB and olibanum extract were simultaneously added into the FBS-free medium. The rings were stained with Diff-Quik, photographed and the length of the sprouts analyzed using Scion Image software on day 5.

Statistical analysis

Data are expressed as means±SEM. The student *t* test was used to compare the data. Findings with p values < 0.05 were considered statistically significant.

RESULTS

Effect of olibanum extract on PDGF-BB-induced migration and proliferation

To examine the effect of olibanum extract, on cellular responses, the effects of the water extract of olibanum was tested on migration and proliferation in response to PDGF-BB in RASMCs using a modified Boyden chamber and the BrdU incorporation assay. PDGF-BB (10 ng/ml) increased RASMC migration (214.1±7.3% of control). Olibanum extract (1~1,000 μg/ml) inhibited PDGF-BB (10 ng/ml)-stimulated migration in a dose-dependent manner; this response was maximum at the 1,000 μg/ml concentration (84.9±7.5% of control, n=8; Fig. 1). PDGF-BB (10 ng/ml) also increased the proliferation of RASMCs (333.3±37.1% of control); this response was inhibited by treatment with olibanum (1~1,000 μg/ml) in a dose dependent fashion. This inhibitory effect was significant at a concentration of 100 μg/ml (117.8±9.3% of control) and reached a maximum at 1,000 μg/ml, which was associated with a level lower than observed at the basal level of RASMC proliferation (43.3±16.7% of control, n=10; Fig. 2). In addition, we attempted to confirm whether the decrease in the RASMC proliferation, below basal levels in response to olibanum, resulted from its cytotoxic effects on the cells. However, the RASMC viability, using the XTT assay, was not altered by olibanum (1~1,000 μg/ml; n=8; data not shown).

Effect of olibanum extract on PDGF-BB-induced phosphorylation of kinases in RASMCs

PDGF-induced vascular smooth muscle cell migration and proliferation has been shown to be mediated by MAPKs and many other kinases (Bilato et al., 1995; Liu et al., 2007). To determine the mechanism of the inhibitory effects of olibanum, on vascular responses, the RASMCs were stimulated with PDGF-BB after dose-dependent treatment with olibanum extract. PDGF-BB (10 ng/ml) increased the phosphorylation of p38 MAPK (312±19.0% of control). PDGF-BB-stimulated phosphorylation of p38 MAPK was inhibited by olibanum extract in a dose-dependent manner (1~1,000 μg/ml) (n=4; Fig. 3); this was significant starting at an olibanum concentration of 10 μg/ml (169.4±20.4% of control), and reached a maximum at 1000 μg/ml (81.4±37.2% of control). On the other hand, the ERK1/2 phosphorylation induced by PDGF-BB (10 ng/ml) did not alter the RASMCs treated with olibanum extract (1~1,000 μg/ml) (n=4; Fig. 3). It is known that Hsp27 acts as a downstream molecule to p38 MAPK and participates in vascular cell migration (Lee et al., 2007). The effects of olibanum on the phosphorylation of Hsp27 in response to PDGF-BB were examined. PDGF-BB (10 ng/ml) increased the phosphorylation of Hsp27 (326.4±3.5% of control, n=4), which was maximally inhibited by treatment with 1,000 μg/ml of olibanum (101.3±36.9% of control, n=4; Fig. 4).

The role of olibanum extract in PDGF-BB-stimulated sprout growth of aortic rings

To investigate the role of olibanum extract *ex vivo*, the effect of olibanum extract was examined using the aortic ring assay in Matrigel. The sprout outgrowth of the aortic rings was increased by treatment with 10 ng/ml of PDGF-BB (436.2±54.5% of control). Pretreatment of the RASMCs with olibanum extract (1~1,000 μg/ml) diminished the PDGF-BB-increased formation of aortic sprouts

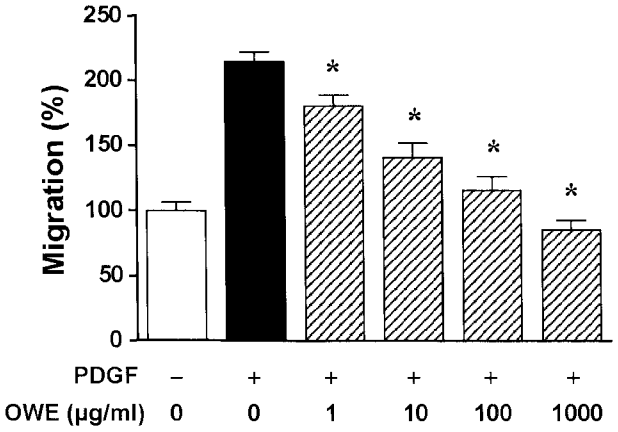

Fig. 1. Effect of olibanum extract on PDGF-BB-stimulated RASMC migration. The effect of olibanum extract on RASMC migration. The cells from SD rats were treated with or without olibanum extract (1~1,000 μg/ml) or PDGF-BB (10 ng/ml) for 90 min. Migration was quantified using a Boyden chamber assay as described in the Methods section. Cell migration in the quiescent state was expressed as 100% (n=8). Each value represents the mean±SEM. *Denotes a significant difference from the PDGF-BB-stimulated state, with $p<0.05$.

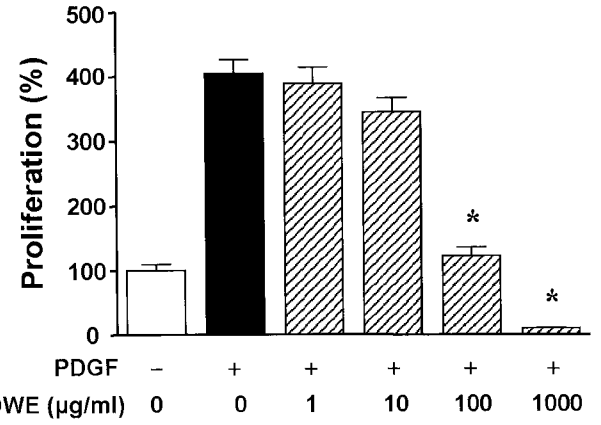

Fig. 2. Effect of olibanum extract on PDGF-BB-stimulated RASMC proliferation. Cells were treated with or without olibanum extract (1~1,000 μg/ml) and then stimulated by PDGF-BB (10 ng/ml) for 36 h. Cell proliferation was examined with the BrdU incorporation assay. Proliferation in the quiescent state was considered as 100% (n=10). Each value represents the mean±SEM. *Significant differences from responses in the PDGF-BB-stimulated state, with $p<0.05$.

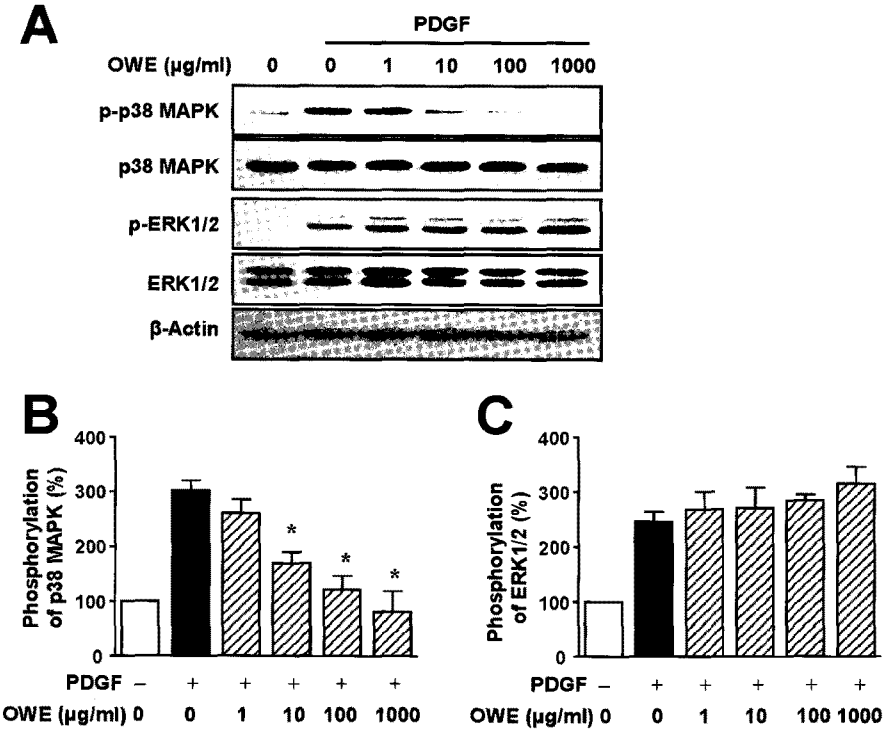

Fig. 3. Effects of olibanum extract on activity of MAPK in RASMCs. After cells were treated without or with olibanum extract (1~1,000 μg/ml) for 30 min, cells were stimulated with PDGF-BB (10 ng/ml) for 10 min. The RASMC lysates were immunoblotted with antibodies. The phosphorylation of p38 MAPK and ERK1/2 was examined using phosphor-specific antibodies. The total expression of kinases and β-actin was measured using non-phospho-specific antibodies and anti-β-actin antibody, respectively. (B, C) Statistical analysis of the phosphorylation level of MAPKs obtained in (A). The basal levels of phosphorylation are considered as 100% (n=4). Each value represents the mean±SEM. *Significant differences from the PDGF-BB-stimulated state, with $p<0.05$. p-p38, phosphorylated p38 MAPK; p-ERK1/2, phosphorylated ERK1/2.

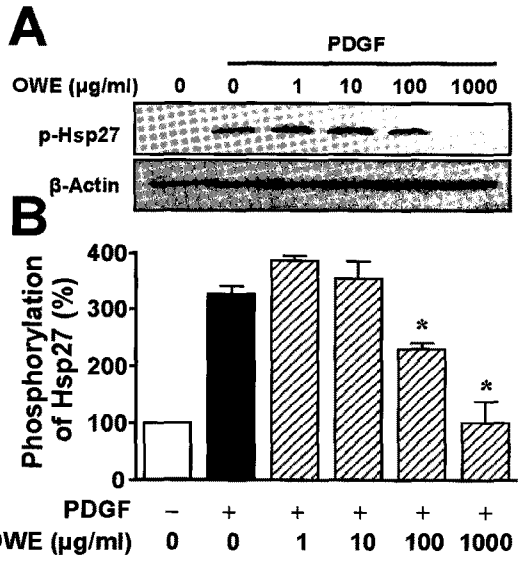

Fig. 4. Effect of olibanum extract on PDGF-BB-induced phosphorylation of Hsp27 in RASMCs. The phosphorylation of Hsp27 was examined using a phospho-specific antibody. (B) Statistical analysis of the phosphorylation level of Hsp27 obtained in (A). The basal levels of phosphorylation are considered as 100% (n=4). Each value represents the mean±SEM. *Significant differences from the PDGF-BB-stimulated state, with $p<0.05$. p-Hsp27, phosphorylated Hsp27.

in a dose-dependent manner. This inhibitory response reached a maximum at a 1,000 μg/ml concentration of olibanum (179.0±34.6% of control, n=4; Fig. 5).

DISCUSSION

The effects of olibanum in various disorders have been widely studied with extracts isolated with organic solvents including MeOH, EtOH and Hexane in herbal medicines; these compounds have been compared with the water-soluble extracts used commonly in Indian ayurvedic medicine and oriental medicine (Huang et al., 2000; Chevrier et al., 2005; Y J et al., 2006). Based on the positive results in animal studies and clinical trials of the European Medicine Agency in 2002, olibanum extract was designated orphan drug status for the treatment of the peritumor edema associated with brain tumors (Winking et al., 2000). It has been reported that olibanum extract affects the migration and proliferation of cells including cancer cells and lymphocytes (Sharma et al., 1988; Gayathri et al., 2007). These findings have suggested that olibanum extract exerts an inhibitory effect on vascular smooth muscle cell behavior. The goal of this study was to investigate the effects of olibanum on RASMC responses with olibanum extract isolated using a water extraction method. The results of this study demonstrate, for the first time, that olibanum extract inhibits PDGF-induced migration and proliferation in rat aortic smooth muscle cells. These effects were confirmed by the findings of the *ex vivo* analysis of the formation of sprouts from aortic rings. Our results suggest that olibanum extracts might be considered a candidate agent for the inhibition of vascular smooth muscle cell migration and proliferation.

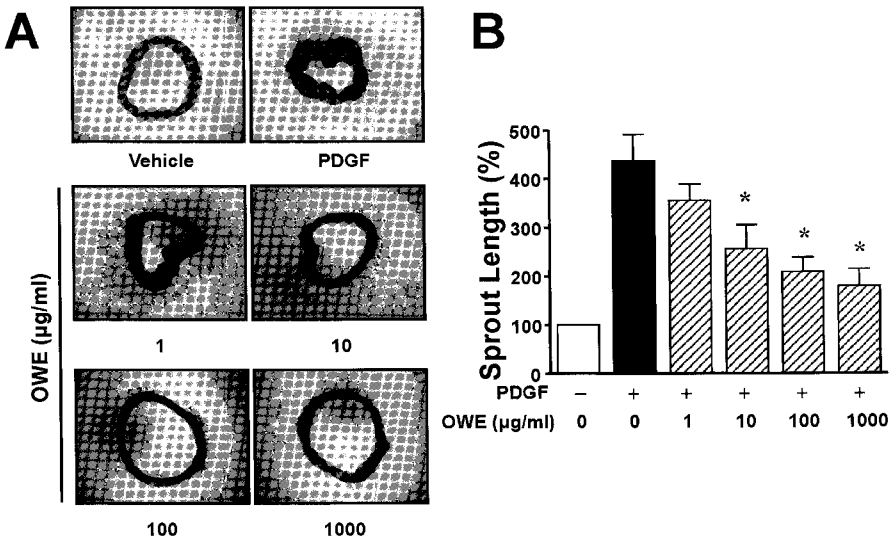

Fig. 5. *Ex vivo* analysis of olibanum extract on sprout formation of aortic rings in response to PDGF-BB. Aortic rings (1 mm) were embedded in Matrigel and cultured. Rings without any treatment (vehicle) and PDGF-BB (10 ng/ml) or olibanum extract (1~1,000 μg/ml)-treated rings. Results were obtained on day 5. (B) The statistical results obtained from panel (A). The level in vehicle (0.1% DMSO)- treated rings is expressed as 100% (n=4). Each value represents the mean±SEM. *Denotes a significant difference from the PDGF-BB (10 ng/ml)-stimulated state, with $p<0.05$.

MAPK pathways play an important role in a variety of cell functions. Proliferation and migration of RASMCs are critical events in vascular disorders such as atherosclerosis and restenosis (Ranies and Ross, 1993), and are associated with MAPKs. PDGF is known to play a central role in the pathogenesis of various vascular disorders (Pompili et al., 1995). PDGF enhances the phosphorylation of p38 MAPK and ERK1/2, and increases the migration and proliferation of RASMCs (Lee et al., 2007). Moreover, it has recently been reported that the olibanum extract from *Boswellia serrata* inhibits the LPS-induced phosphorylation of MAPKs, JNK and p38 MAPK, in blood mononuclear cells (Gayathri et al., 2007); these findings imply that olibanum can exert an inhibitory effect on the MAPK signaling pathway. In this study, we explored the possible role of p38 MAPK and ERK 1/2, and the effects of olibanum on PDGF-induced RASMC migration and proliferation; JNK, an important isoenzyme of MAPK was not included because these two PDGF-induced RASMC responses (proliferation and migration) were not significantly suppressed by JNK inhibition in previous studies (Lee et al., 2007). The activation of p38 MAPK stimulated various cell functions such as contraction, migration, and proliferation in the vascular smooth muscle cells (Geraldes et al., 2002; Kim et al., 2004). PDGF-induced migration and proliferation was associated with p38 MAPK phosphorylation in RASMCs (Lee et al., 2007). The findings of this study showed that olibanum extract inhibited the PDGF-induced phosphorylation of p38 MAPK in the RASMCs and attenuated RASMC migration induced by PDGF stimulation. Therefore, our data suggest that the suppression of p38 MAPK activity may be responsible for the inhibition of PDGF-increased RASMC migration and proliferation in response to olibanum extract. However, olibanum-induced interaction between the p38 MAPK activity and the two cellular responses, migration and proliferation, in the RASMCs, was not directly demonstrated in this study.

Hsp27 has been reported to be an actin binding protein that regulates its polymerization, and is expressed at high levels in smooth muscle (Guay et al., 1997). The signaling pathway involved in Hsp27 activity is known to be important in actin cytoskeleton remodeling and has been associated with migration in smooth muscle (Hedges et al., 1999). PDGF induced the phosphorylation of p38 MAPK and Hsp27, and p38 MAPK phosphorylated Hsp27 in smooth muscle cells (Kozawa et al., 1999); these results suggest that p38 MAPK is an upstream molecule with respect to Hsp27. Similarly, in a recent study from this lab, we reported that the phosphorylation of Hsp27 was enhanced by PDGF in RASMCS, which was attenuated by a p38 MAPK inhibitor, and PDGF-induced migration was inhibited by the treatment of a p38 MAPK inhibitor and the small interfering RNA of Hsp27 (Lee et al., 2007). Moreover, it has been reported that Hsp27 promotes proliferation in vascular smooth muscle cells (Champagne et al., 1999). In the present study, we found that the PDGF-induced phosphorylation of Hsp27 was attenuated by treatment with olibanum extract. Therefore, the findings of this study suggest that the olibanum extract-induced inhibition of PDGF-stimulated RASMC migration and proliferation may be associated with the p38 MAPK-mediated Hsp27 pathway.

Among the three MAPKs, ERK1/2 is known to have a significant impact on PDGF-induced cell growth (Zhan et al., 2003). The phosphorylation level of ERK1/2 has been shown to be enhanced by PDGF stimulation. The stimulated migration and proliferation in response to PDGF, in the vascular smooth muscle cells, was attenuated by inhibitors of ERK1/2 (Dardick et al., 2005; Lee et al., 2007). In the present study, PDGF increased the phosphorylation of ERK1/2 in the RASMCs, and this response was not inhibited by treatment with olibanum extract. Similarly, a previous report demonstrated that olibanum extract did not induce ERK1/2 phosphorylation in response to LPS, in different cells than the type used in this study (Gayathri et al., 2007), Therefore, it is possible that olibanum extract does not affect the ERK1/2 activity in RASMCs, and this kinase pathway might not be involved in the olibanum extract inhibition of PDGF-induced migration and proliferation of the RASMCs. These results suggest that MAPK isoforms may be regulated differently by olibanum extract.

In summary, the results of the present study demonstrated, for the first time that PDGF-induced migration and proliferation in RASMCs was inhibited by olibanum extract treatment, and that olibanum extract attenuated the

PDGF-enhanced phosphorylation of p38 MAPK and Hsp27. However, the level of phosphorylation of ERK1/2, enhanced by PDGF, was not altered by olibanum. These data provide evidence that olibanum extract exerts inhibitory effects on PDGF-induced migration and proliferation in RASMCs, and that these inhibitory effects may be induced by the p38 MAPK and/or Hsp27 pathways. The findings of this study, therefore, may provide valuable information for additional research or drug development for the prevention and treatment of vascular diseases such as atherosclerosis. However, further study will be required for elucidating the mechanism linking olibanum extract and PDGF-stimulated signals, in the p38 MAPK and Hsp 27 pathways, associated with RASMC migration and proliferation. It appears that olibanum extract targets the cell surface because the olibanum extract used in this study was water soluble; this might provide an important clue as to the mechanism involved in the inhibitory effects of olibanum extract on RASMC migration and proliferation in response to PDGF. Further studies are needed to clarify the mechanisms.

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