Magnobovatol inhibits smooth muscle cell migration by suppressing PDGF-Rβ phosphorylation and inhibiting matrix metalloproteinase-2 expression

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Abstract. The migration of vascular smooth muscle cells (VSMCs) may play a crucial role in the pathogenesis of vascular diseases, such as atherosclerosis and post-angioplasty restenosis. Platelet-derived growth factor (PDGF)-BB is a potent mitogen for VSMCs and plays an important role in the intimal accumulation of VSMCs. Magnobovatol, a new neolignan from the fruits of *Magnolia obovata*, has been shown to have anticancer properties. However, the effects of magnobovatol on VSMCs are unknown. In the present study, we examined the effects of magnobovatol on the PDGF-BB-induced migration of mouse and human VSMCs, as well as the underlying mechanisms. Magnobovatol significantly inhibited the PDGF-BB-induced migration of mouse and human VSMCs without inducing cell death (as shown by MTT assay and wound healing assay). Additionally, we demonstrated that magnobovatol significantly blocked the PDGF-BB-induced phosphorylation of the PDGF receptor (PDGF-R), Akt and extracellular signal-regulated kinase (ERK)1/2 by inhibiting the activation of the PDGF-BB signaling pathway. Moreover, in both mouse and human VSMCs, magnobovatol inhibited PDGF-induced matrix metalloproteinase (MMP)-2 expression at the mRNA and protein level, as well as the proteolytic activity of MMP-2 (as shown by western blot analysis, RT-PCR, gelatin zymography and ELISA). In addition, the sprout outgrowth formation of aortic rings induced by PDGF-BB was inhibited by magnobovatol (as shown by aortic ring assay). Taken together, our findings indicate that magnobovatol inhibits VSMC migration by decreasing MMP-2 expression through PDGF-R and the ERK1/2 and Akt pathways. Our data may improve the understanding of the anti-atherogenic effects of magnobovatol in VSMCs.

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Introduction

Vascular injury induces cellular responses which lead to clinical events, including atherosclerosis, hypertension and restenosis (1). One common feature of vascular lesions is the migration of vascular smooth muscle cells (VSMCs) (2). VSMCs are mostly found in the media layer of normal arteries; however, VSMCs migrate from the media towards the intima during the development of atherosclerosis or vascular damage (3,4). A number of factors, including matrix metalloproteinases (MMPs), growth factors, cytokines and chemokines affect VSMC migration in the microenvironment of atherosclerotic lesions (5). Therefore, the inhibition of VSMC migration represents a potentially important therapeutic strategy for the treatment of cardiovascular diseases.

Platelet-derived growth factor (PDGF), a potent chemoattractant for VSMCs, is encoded by 4 genes: PDGF-A, -B, -C and -D (6). Among these, only PDGF-BB can bind to all homo- or heterodimeric PDGF receptors (PDGF-Rs) (7). The binding of PDGF-BB to PDGF-R activates various downstream signaling molecules, including those in the phosphatidylinositol 3 kinase (PI3K)/Akt, phospholipase C (PLC)-γ1, and extracellular signal-regulated kinase (ERK)1/2 pathways (1,8,9). Akt is a downstream target of PI3K and plays a pivotal role in cell migration, growth and anti-apoptotic events in various cell types (10,11). The mitogen-activated protein kinase (MAPK) signaling pathway also plays an important role in the regulation of the proliferation, migration and survival of mammalian cells (8,12). Consequently, the upregulation of PDGF signaling can cause the development and progression of cardiovascular diseases, such as hypertension and atherosclerosis (13). Therefore, modulating the PDGF signaling pathway

in VSMCs may be a favorable pharmacological strategy for the prevention of atherosclerosis.

Various herbs and plants have traditionally been used in oriental folk medicine for the treatment of various diseases. The bark of *Magnolia obovata* Thunb. (Magnoliaceae; *M. obovata*), also known as Hu-Bak, is one of the most popular therapeutics in traditional Korean medicine and is widely used to treat fever, headaches, diarrhea, anxiety and to relieve asthma (14,15). It contains many types of secondary metabolites, such as neolignans, sesquiterpene-neolignans, aporphine alkaloids and essential oils (16,17). These compounds show anti-gastric ulcer, anti-platelet, cytotoxic, anti-inflammatory, and anti-complement activities (18,19). Recently, magnobovatol, a newly identified neolignan from the fruits of *M. obovata*, has been reported to inhibit the growth of a variety of human cancer cells, including breast, colorectal, melanoma cancer cell lines (20). However, the effects of magnobovatol on smooth muscle cell migration have not yet been elucidated, at least to the best of our knowledge. Thus, the aim of this study was to elucidate the anti-migratory activity and the mechanistic target of magnobovatol in PDGF-BB-stimulated VSMCs.

Materials and methods

Cell culture. Human aortic smooth muscle cells (HASMCs) were purchased from Applied Biological Materials Inc. (Cat. no. T4050) and were maintained in Prigrow III medium (Applied Biological Materials Inc.) (Cat. no. TM003) in the presence of 5% FBS (Cat. no. SH30919.03; HyClone, Logan, UT, USA) and penicillin (100 U/ml)/streptomycin (100 μg/ml) (Invitrogen, Carlsbad, CA, USA). MOVAS-1 murine primary aortic VSMCs were obtained from the American Type Culture Collection (ATCC; Cat. no CRL-2797™) and were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% FBS, G418 (0.2 mg/ml), penicillin (100 U/ml)/streptomycin (100 μg/ml) at 37˚C with 5% CO_2 atmosphere in a humidified incubator.

Reagents. Magnobovatol was kindly provided by Dr Nam-In Baek (Department of Oriental Medicine Biotechnology, Kyung Hee University, Yongin, Korea) (16). The structure of magnobovatol is illustrated in Fig. 1A. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Sigma (St. Louis, MO, USA). PDGF-BB was obtained from Peprotech (Seoul, Korea). Gelatin was obtained from Difco (Lexington, KY, USA). Lipofectamine 2000 reagent was purchased from Invitrogen. Anti-p-PDGFRβ (Cat. no. 4549) and anti-p-Akt (Ser473; Cat. no. 4058) antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-p-ERK (Cat. no. sc-7383), anti-ERK (Cat. no. sc-93), anti-Akt (Cat. no. sc-5298) and anti-α-tubulin (Cat. no. sc-5286) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All the chemicals not mentioned above were obtained from Sigma.

Cell viability. Cell viability was measured by MTT assay. The cells were seeded in 96-well plates at a density of $2x10^5$ cells/well. Following serum starvation for 24 h, the cells were treated with PDGF-BB (10 ng/ml) in the presence of 1-50 μg/ml magnobovatol for 30 min. Following incubation for 24 h at 37˚C, 20 μl of 5 mg/ml MTT solution were added and the cells were incubated for 1 h at 37˚C and 100 μl dimethyl sulfoxide was added to each well to dissolve the formazan. The absorbance was measured at 550 nm using a microplate reader (Magellan; Tecan). Cell viability is expressed as a percentage of the absorbance value determined for the control cultures.

Western blot analysis. To examine the protein expression patterns, equal amounts (15 μg) of total protein extracts were prepared. The cell lysates were seperated on 9% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The blots were then incubated with the antibodies (anti-p-PDGF-R, anti-p-ERK, anti-ERK, anti-p-Akt and anti-Akt antibodies) and detected using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The same blots were then stripped and reprobed with anti-tubulin antibody for use as an internal control. Quantitative analysis of the results of western blot analysis were carried out using ImageJ software.

Wound healing assay. The cells were incubated until they reached 90 to 100% confluence in 12-well plates. Subsequently, a scratch was gently made using a P-10 pipette tip, and the cells were then treated with 1-10 μg/ml magnobovatol with PDGF-BB. The cells were subsequently allowed to migrate [24 h (MOVAS-1) or 48 h (HASMCs)] and incubated for various periods of time and images were acquired. Phase contrast images were acquired using a Nikon microscopy system (Nikon Instruments Inc., Melville, NY, USA). The measurement of the wound healing gap distance was performed using ImageJ software.

RT-PCR. Total RNA was extracted from the cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Approximately 2 μg of total RNA was used to prepare cDNA using the SuperScript First Strand cDNA Synthesis kit (Bioneer Corporation, Daejeon, South Korea). The following primers were used in this study: mMMP-2 forward, 5'-AAGGATGGACTCCTGGCACATGCCTTT-3' and reverse, 5'-ACCTGTGGGCTTGTCACGTGGTGT-3'; mGAPDH forward, 5'-GGAGCCAAAAGGGTCATCAT-3' and reverse, 5'-GTGATGGCATGGACTGTGGT-3'; hMMP-2 forward, 5'-ATGACAGCTGCACCACTGAG-3' and reverse, 5'-ATTTGTTGCCCAGGAAAGTG-3'; and hGAPDH forward, 5'-CCATCACCATCTTCCAGGAG-3' and reverse, 5'-CCTGCTTCACCACGTTCTTG-3'. PCR was performed with Platinum Taq polymerase (Invitrogen) under the following conditions: 30 cycles of 96˚C for 40 sec, 55˚C (MMP-2) or 60˚C (GAPDH) for 40 sec, 72˚C for 1 min followed by 10 min at 72˚C. The PCR products were electrophoresed on a 2% (w/v) agarose gel in 1X Tris-acetate-EDTA (TAE) buffer, and stained with ethidium bromide solution. All the PCR reactions were repeated at least 3 times. GAPDH was amplified as an internal control. The intensity of each band amplified by RT-PCR was analyzed using MultiImage™ Light Cabinet (version 5.5; Alpha Innotech Corp., San Leandro, CA, USA), and normalized to that of GAPDH mRNA in corresponding samples.

Gelatin zymography. The presence of MMP-2 in the supernatants of magnobovatol- and/or PDGF-BB-treated SMCs were analyzed using gelatin zymograms. Briefly, the cells

were incubated in serum-free DMEM and the supernatants were collected following incubation for 24 h at 37˚C, clarified by centrifugation (13,000 rpm for 5 min at 4˚C), normalized to the total protein concentration of the cell lysate, mixed with non-reducing Laemmli smaple buffer, and separated by electrophoresis on 10% SDS-PAGE gels containing 1 mg/ml gelatin (Difco). Following electrophoresis, the gels were re-natured by washing in 2.5% Triton X-100 solution twice for 30 min to remove all the SDS. The gels were then incubated in 50 mmol/l Tris-HCl (pH 7.4), 5 mmol/l $CaCl_2$, and 1 μM $ZnCl_2$ at 37˚C overnight. Following incubation, the gels were stained with 0.05% Coomassie brilliant blue R-250 for 30 min at room temperature and then destained in distilled water. MMP-2 activities were visible as clear bands on a blue background where the gelatin substrate had been hydrolyzed by enzyme activity.

ELISA for MMP-2. The supernatants were collected for measuring the amount of secreted MMP-2 protein. The total MMP-2 protein was assayed according to the instructions provided with the Quantkine ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA). In brief, samples and MMP-2 standards were added to microplates pre-coated with antibody specifically recognizing both the pro- and active forms of MMP-2. After washing, bound MMP-2 was measured using a horseradish peroxidase-conjugated secondary anti-MMP-2 antibody, developed with hydrogen peroxide and tetramethylbenzidine. The optical density was measured at 450 nm using a Bio-Rad Model 550 microplate reader and associated Microplate Manager software (Bio-Rad Laboratories, Mississauga, ON, Canada). The absorbance of the samples was measured at 450 nm using a microplate reader.

Aortic ring assay. The *ex vivo* migration and proliferation of VSMCs were measured by aortic ring assay using Matrigel. Male C57BL/6J mice (n=6 in each group), which were 6-8 weeks of age, were purchased from Orient Bio, Inc. (Seoul, Korea) and housed under special pathogen-free conditions. All animals were treated in accordance with the Animal Care Guidelines of Use of Laboratory Animals and approved by the Laboratory of Animal Research in Asan institute of Life Sciences. For the *ex vivo* migration and proliferation of VSMCs, thoracic aortas were removed from the mice which were sacrificed by prolonged exposure to a dose of isoflurane followed by cervical dislocation and immediately transferred to a culture dish containing serum-free DMEM. The peri-aortic fibroadipose tissue was carefully removed with fine microdissecting forceps and iridectomy scissors, paying special attention not to damage the aortic wall. One millimeter-thick aortic rings, approximately 12 per aorta, were sectioned and extensively rinsed in 5 consecutive washes of serum-free DMEM. These rings were placed and embedded in 48-well plates coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Simultaneously, magnobovatol (0-10 μg/ml) and PDGF-BB (10 ng/ml) were added to the culture medium for 3 days. The ring formation images were acquired using a ZEISS microscope (Carl Zeiss, Oberkochen, Germany) and the length of the sprouts was analyzed.

All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of University of Ulsan College of Medicine (approval no. 2014-12-140) and were performed in strict accordance with the Association for Assessment and Accreditation of Laboratory Animal Care and the NIH guidelines (Guide for the Care and Use of Laboratory Animals).

Statistical analysis. Statistical analysis was performed using the computer program Prism (GraphPad Software, Inc., La Jolla, CA, USA). The results are presented as the means ± SE. The statistical significance of the differences between groups was analyzed by repeated measures of one-way analysis of variance followed by a Student's t-test. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Magnobovatol inhibits PDGF-BB-induced VSMC migration. To assess the effects of magnobovatol on cell proliferation, mouse aortic smooth muscle cells (MOVAS-1 cells) and HASMCs were treated with magnobovatol in the absence or presence of 10 ng/ml PDGF-BB for 24 h. Compared to the untreated control cells, the MOVAS-1 cells (Fig. 1B, black bar) and the HASMCs (Fig. 1B, white bar) treated with magnobovatol at concentrations between 0 and 10 μg/ml exhibited no signs of cytotoxicity, regardless of whether they were treated with PDGF-BB or not. Therefore, this concentration range was used in all the subsequent experiments.

To determine the effects of magnobovatol on VSMC migration, we performed a wound healing assay using both the HASMCs and MOVAS-1 cells. After a scratch wound was made on the cells in the plates, the effects of magnobovatol various concentrations on PDGF-BB-induced cell migration were examined. The cells were allowed to migrate for 24 h (MOVAS-1) or 48 h (HASMCs), and the migration distances were then measured. As shown in Fig. 1C and D, magnobovatol suppressed the PDGF-BB-induced migration of both the HASMCs (Fig. 1C) and the MOVAS-1 cells (Fig. 1D) in a dose-dependent manner, demonstrating that magnobovatol suppressed VSMC migration.

Magnobovatol inhibits the activation of PDGF-R signaling and the ERK1/2 and Akt signaling pathways. PDGF triggers the PI3K/Akt and Ras/MAPK signaling cascades by activating PDGF-R (8). Thus, in this study, to determine whether magnobovatol inhibits PDGF-induced mitogenic effects, we treated the HASMCs (Fig. 2A and B) and MOVAS-1 cells (Fig. 2C and D) with 10 μg/ml magnobovatol followed by treatment with PDGF-BB. PDGF-BB markedly induced the phosphorylation of PDGF-R, which was substantially attenuated by treatment with magnobovatol (Fig. 2). In addition, magnobovatol inhibited PDGF-induced ERK1/2 and Akt phosphorylation in both the HASMCs (Fig. 2A and B) and MOVAS-1 cells (Fig. 2C and D). These results indicate that magnobovatol regulates VSMC migration through the inhibition of PDGF-R activation.

Magnobovatol suppresses PDGF-BB-induced MMP-2 expression and proteolytic activity. MMPs involved in the breakdown of the extracellular matrix (ECM) are associated with the development of atherosclerosis (21). In particular, the gelatinase MMP-2 is predominantly expressed in VSMCs in advanced atherosclerotic lesions and in injury-induced neointimal lesions (22). Since PDGF is a potent inducer of MMP-2 (23), in this study, we examined the effects of magnobovatol on the expression of MMP-2 in VSMCs. Our results revealed that the basal levels of MMP-2

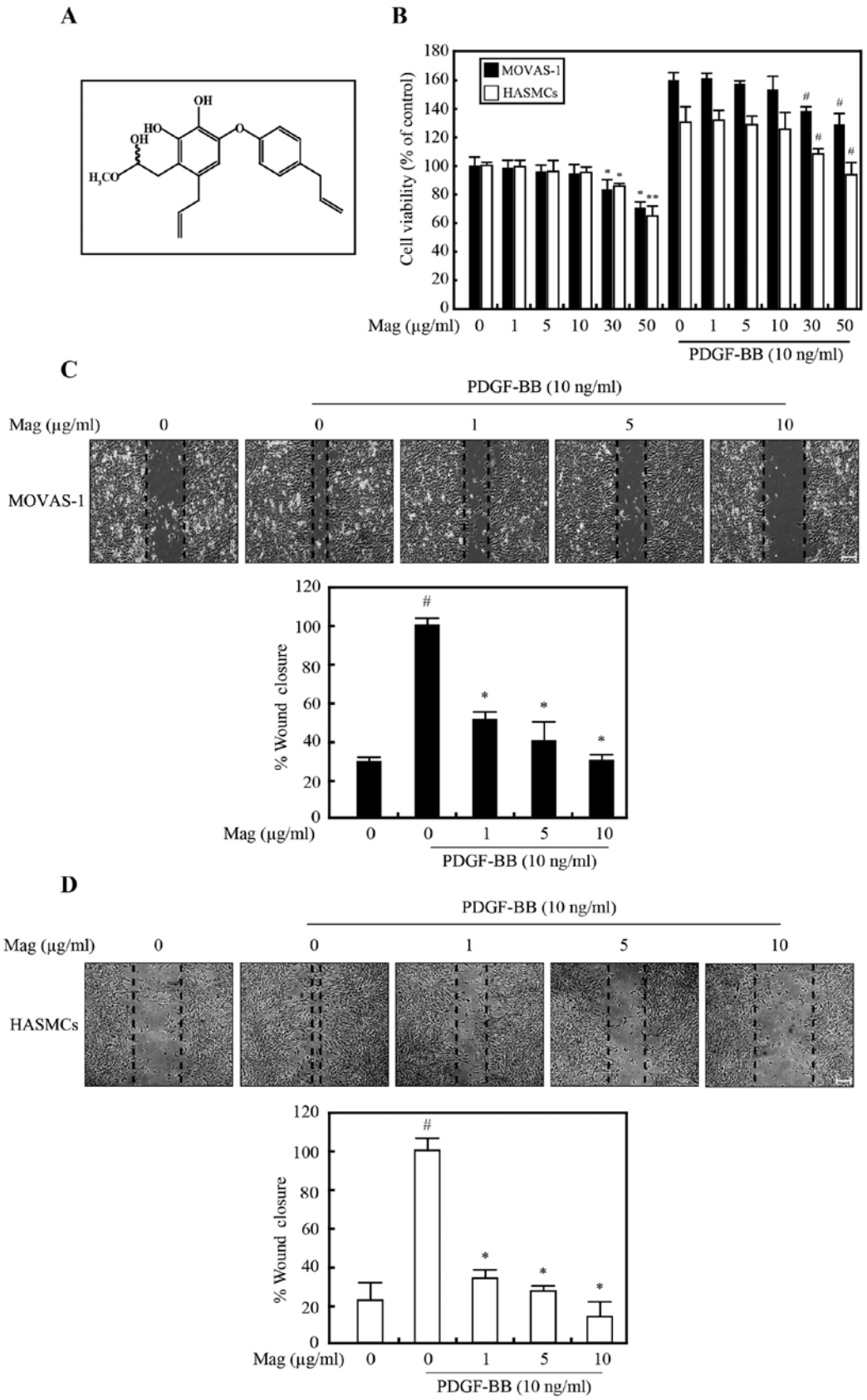

Figure 1. Magnobovatol inhibits the platelet-derived growth factor (PDGF)-BB-induced migration of human aortic smooth muscle cells (HASMCs) and MOVAS-1 cells. (A) Chemical structure of magnobovatol. (B) HASMCs (white bars) and MOVAS-1 cells (black bars) pre-treated with 0-50 μg/ml magnobovatol were stimulated with or without 10 ng/ml PDGF-BB for 24 h. Cell viability was measured by MTT assay; *P<0.05, **P<0.01 vs. untreated control cells, $^{\#}$P<0.01 vs. PDGF-treated cells. Data represent the means ± SE of 3 independent experiments. A scratch was formed on the (C) HASMCs and (D) MOVAS-1 cells using a pipette tip and the cells were incubated with various concentrations of magnobovatol in serum-free medium. After 30 min, the cells were stimulated with 10 ng/ml PDGF-BB for 24 h (MOVAS-1 cells) or 48 h (HASMCs). Representative images of wound healing were taken at the time of scratching and 24 h (or 48 h) after wounding. Original magnification, x100. Scale bar, 100 μm. Wound healing was quantified as the percentage of cells migrating into the wound with respect to the total number of cells. $^{\#}$P<0.001 vs. untreated control cells, *P<0.01 vs. PDGF-treated cells. Data represent the mean ± SE of 3 independent experiments.

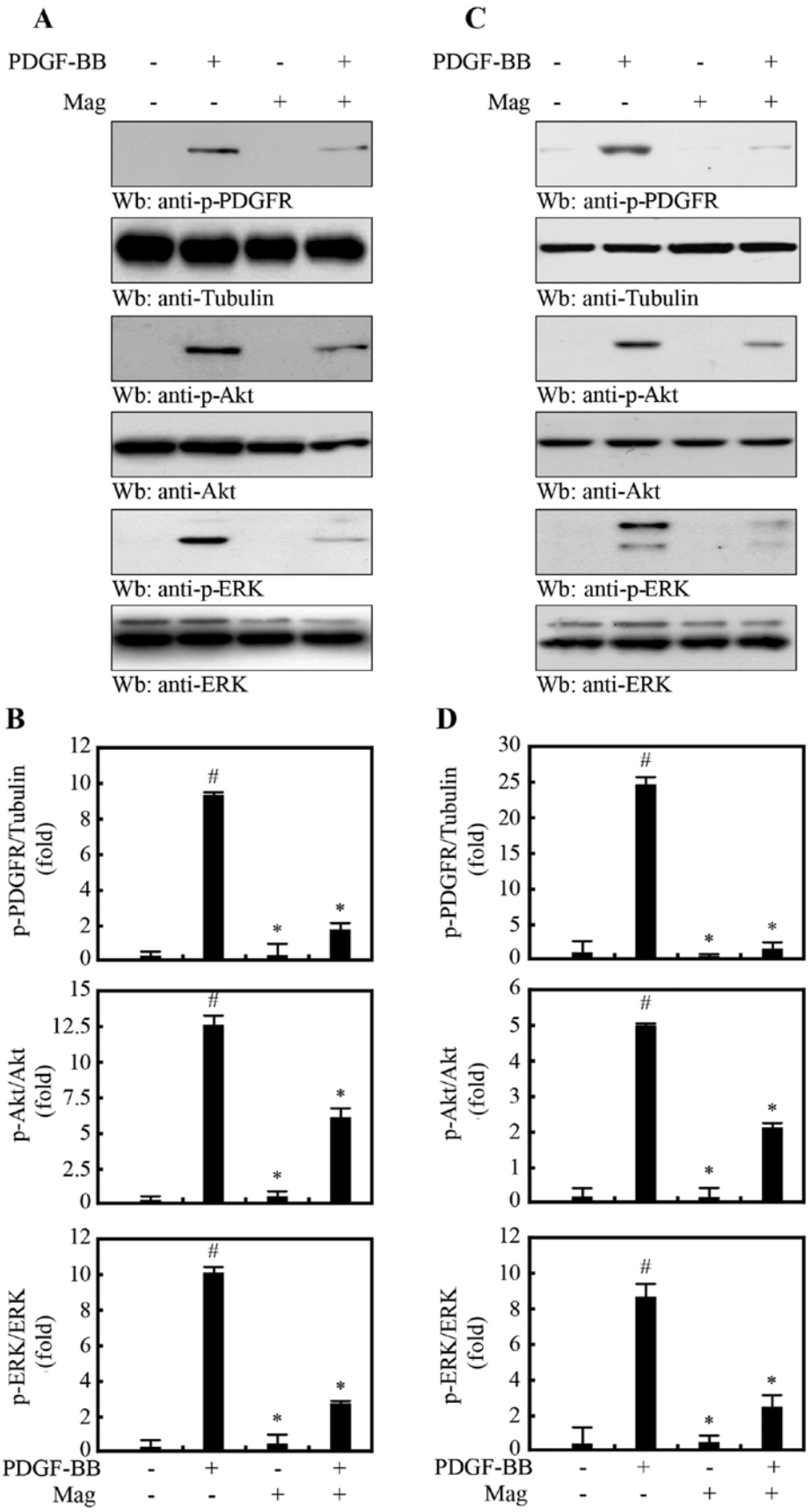

Figure 2. Effect of magnobovatol on the platelet-derived growth factor (PDGF)-induced activation of downstream signaling pathways. (A and B) Human aortic smooth muscle cells (HASMCs) and (C and D) MOVAS-1 cells were treated with magnobovatol for 30 min and then stimulated with PDGF-BB for 15 min. Cell lysates were separated on a 10% SDS-polyacrylamide gel and subjected to western blot analysis with the indicated antibodies. The membrane was stripped and reprobed with anti-tubulin, -Akt, and -extracellular signal-regulated kinase (ERK) antibodies as loading controls. Relative protein levels were quantified by densitometric scanning and normalized to the corresponding total protein from 3 independent experiments. $^{\#}$P<0.001 vs. untreated control cells, *P<0.01 vs. PDGF-treated cells. Data represent the means ± SE of 3 independent experiments.

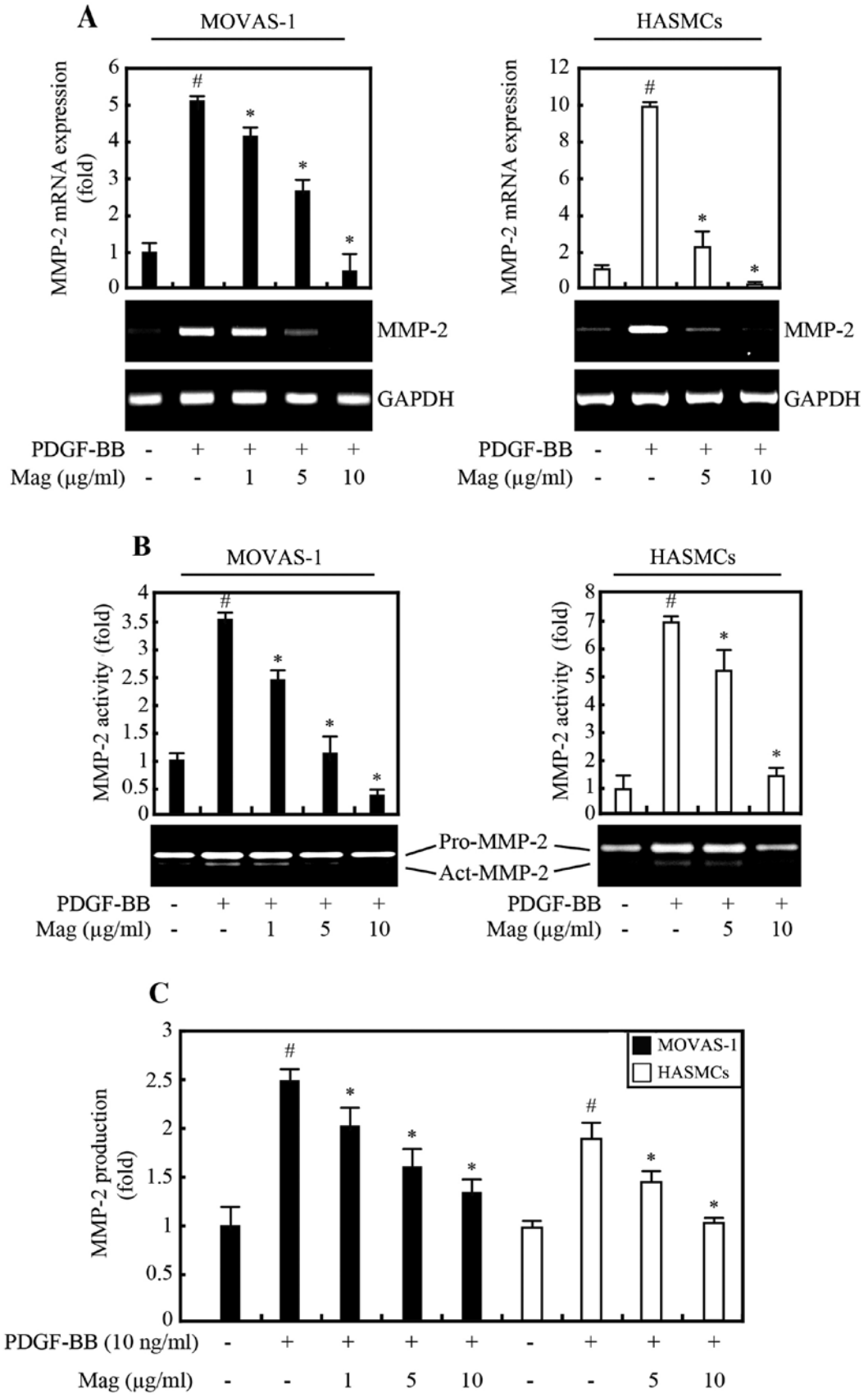

Figure 3. Magnobovatol reduces the platelet-derived growth factor (PDGF)-induced expression and activity of MMP-2 in human aortic smooth muscle cells (HASMCs) and MOVAS-1 cells. (A) HASMCs and MOVAS-1 cells were incubated with the indicated concentrations of magnobovatol for 30 min followed by stimulation with 10 ng/ml PDGF-BB for 24 h. The mRNA level of endogenous MMP-2 was measured by RT-PCR. GAPDH was used as an internal control. (B and C) HASMCs and MOVAS-1 cells were treated with magnobovatol for 30 min and then stimulated with 10 ng/ml PDGF-BB for 24 h. The conditioned medium was collected and assayed for secreted MMP-2 using (B) gelatin zymography and (C) ELISA. $^{\#}$P<0.001 vs. untreated control cells, *P<0.01 vs. PDGF-treated cells. Data represent the means ± SE of 3 independent experiments.

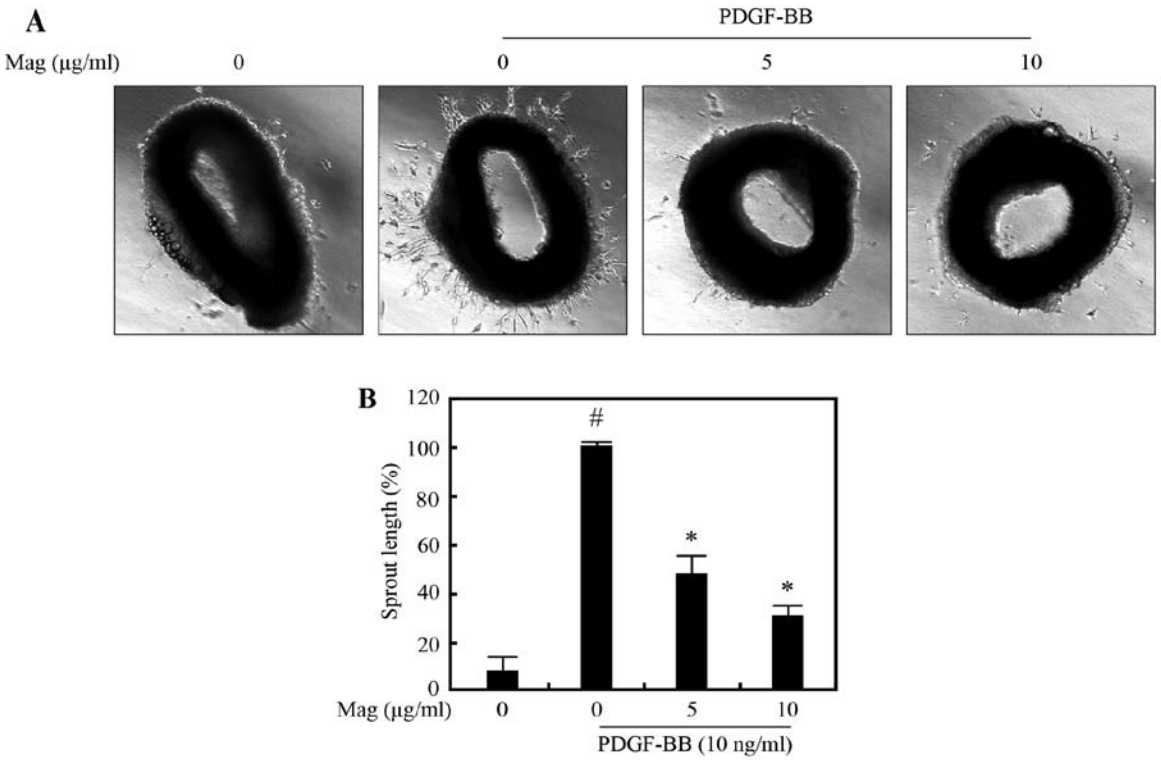

Figure 4. Magnobovatol attenuates platelet-derived growth factor (PDGF)-BB-induced human aortic smooth muscle cell (HASMC) sprout formation of aortic rings. Sprout formation was determined by aortic ring assay *ex vivo*. Aortic rings were placed and embedded in Matrigel in 48-well plates and treated with magnobovatol at the indicated concentrations and PDGF-BB in culture medium for 3 days. (A) Light microscopic images showing the effects of magnobovatol on PDGF-BB induced sprout formation. (B) Quantitative analysis of the aortic ring assay. Quantification is relative to the controls, and the data represent the means ± SE of 3 experiments, $^{\#}P<0.001$ vs. untreated control cells, $^{*}P<0.01$ vs. PDGF-treated cells.

expression and activity were low in the both MOVAS-1 cells and HASMCs, as indicated by RT-PCR (Fig. 3A), gelatin zymography (Fig. 3B), and ELISA (Fig. 3C). However, MMP-2 mRNA expression and protein secretion were markedly increased by stimulation with PDGF-BB (Fig. 3). Of note, magnobovatol inhibited the increase in both MMP-2 expression and secretion, but it had no effect on the expression of MMP-9 (data not shown). These results suggested that the PDGF-BB-induced increase in MMP-2 expression and secretion in VSMCs are inhibited by magnobovatol.

Magnobovatol attenuates PDGF-BB-induced VSMC sprout outgrowth formation of aortic rings. To examine the effects of magnobovatol on VSMC migration in an animal model of atherosclerosis, we performed an *ex vivo* aortic ring assay. Aortic rings isolated from C57BL/6 mice were embedded in Matrigel and the lengths of outgrowth sprouts were measured after 3 days. As shown in Fig. 4, exposure to PDGF-BB (10 ng/ml) increased sprouting, which was significantly attenuated by magnobovatol treatment in a dose-dependent manner. These results suggest that magnobovatol markedly attenuates PDGF-BB-induced VSMC sprout outgrowth formation *ex vivo*.

Discussion

In the present study, we demonstrated the following: i) magnobovatol inhibits PDGF-induced VSMC migration, ii) magnobovatol inhibits the activation of PDGF-R downstream signaling pathways, iii) magnobovatol regulates MMP-2 expression and activity, and iv) consequently, magnobovatol inhibits PDGF-induced migration through the suppression of PDGF-R-mediated MMP-2 expression and proteolytic activity in VSMCs.

PDGF acts as a potent inducer of VSMC proliferation and migration through the phosphorylation of PDGF-R (24). Enhanced PDGF signaling has been implicated in the pathophysiology of a variety of diseases involving vascular wall remodeling (13,25). In the arteries, PDGF-R expression is substantially induced following endothelial injury by angioplasty or early-stage atherosclerosis (26). Conversely, inhibiting the PDGF pathway using antisense oligonucleotides or a blocking antibody has been successfully applied to suppress intimal thickening (27). Treatment with PDGF has previously been demonstrated to induce the phosphorylation of Akt and ERK1/2 in both HASMCs and MOVAS-1 cells (4). In accordance with these findings, we found that magnobovatol significantly attenuated PDGF-BB-induced PDGF-R, Akt and ERK1/2 phosphorylation in VSMCs. These results suggest that PDGF-R may be a direct target of magnobovatol in the inhibition of VSMC migration. Furthermore, PI3K/Akt contributes to ECM destruction by increasing the production of MMP-2 in VSMCs (28). MMP-2 expression is associated with the induction of SMC hyperplasia during atherosclerosis and restenosis. In addition, MMP-2 plays a role in the proliferation and migration of SMCs, which contributes to the intimal thickening of

vascular lesions *in vivo* (29,30). Our results revealed that pre-treatment with magnobovatol inhibited the PDGF-induced increase in MMP-2 expression and proteolytic activity in a dose-dependent manner. Presumably, the suppressive effects of magnobovatol on the PDGF-induced activation of Akt was associated with the subsequent decrease in the mRNA expression and secretion of MMP-2.

In conclusion, the results of our study demonstrated showed that magnobovatol inhibited the migration of cultured VSMCs, which may be major inducers of atherosclerosis and restenosis, *in vitro*. We demonstrate that magnobovatol regulates molecules involved in the remodeling of the vessel wall and/or the accumulation of cells and ECM. Therefore, our data may provide a novel strategy with which to inhibit VSMC migration under pathological conditions, thereby offering a novel therapeutic approach for the treatment of restenosis and other vascular diseases involving VSMC migration.

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