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Therapeutic effect of magnesium lithospermate B on neointimal formation after balloon-induced vascular injury

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Abstract

Vascular smooth muscle cell (VSMC) proliferation and migration in response to platelet-derived growth factor (PDGF) play an important role in the development of atherosclerosis and restenosis. Recent evidence indicates that PDGF increases intracellular levels of reactive oxygen species in VSMCs and that both PDGF-induced VSMC proliferation and migration are reactive oxygen species-dependent. Danshen is a representative oriental medicine used for the treatment of vascular disease. Previously, we reported that magnesium lithospermate B, an active component of Danshen, is a potent antioxidant. Thus we investigated the therapeutic potential of magnesium lithospermate B in neointimal formation after carotid artery injury in rats along with its effects on the PDGF signaling pathway for stimulating VSMC proliferation and migration *in vitro*. PDGF is dimeric glycoprotein composed of two A or two B chains. In this study, we used PDGF-BB, which is one of the isoforms of PDGF (i.e., PDGF-AA, PDGF-BB, and PDGF-AB). Our results demonstrated that magnesium lithospermate B directly scavenged reactive oxygen species in a xanthine/xanthine oxidase system and reduced PDGF-BB-induced intracellular reactive oxygen species generation in VSMCs. In a rat carotid artery balloon injury model, magnesium lithospermate B treatment (10 mg/kg/day, i.p) showed a significant effect on the prevention of neointimal formation compared with vehicle treatment. In cultured VSMCs, magnesium lithospermate B significantly attenuated PDGF-BB-induced cell proliferation and migration as measured by 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2-tetrazolium bromide (MTT) assay and transwell migration assays, respectively. Further, magnesium lithospermate B inhibited PDGF-BB-induced phosphorylation of phospatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathways by scavenging reactive oxygen species. Together, these data indicated that magnesium lithospermate B, a potent reactive oxygen species scavenger, prevented both injury-induced neointimal formation *in vivo* and PDGF-BB-induced VSMC proliferation and migration *in vitro*, suggesting that magnesium lithospermate B may be a promising agent to prevent atherosclerosis and restenosis following angioplasty.

Keywords: Magnesium lithospermate B; Vascular smooth muscle cell; Platelet-derived growth factor; Reactive oxygen species; Neointimal formation

1. Introduction

Vascular smooth muscles in the tunica media are quiescent under the physiological conditions; however, following endothelial injury, they proliferate and migrate to the intima, leading to neointimal hyperplasia and resulting in atherosclerosis and

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restenosis (Schwartz et al., 1992; Schwartz, 1997; Dzau et al., 2002). These processes are triggered by multiple factors including cytokines and growth factors such as platelet-derived growth factor (PDGF), which is produced by platelets, vascular smooth muscle cells (VSMCs), and endothelial cells in the injured vascular wall (Heldin and Westermark, 1999; Leppanen et al., 2000; Miyauchi et al., 1998). The PDGF receptor is expressed at low levels in arteries in healthy adults, but its expression is upregulated during endothelial injury after angioplasty or in the early stage of atherosclerosis (Majesky et al., 1990).

Several studies have reported that reactive oxygen species generation is increased during restenosis after angioplasty (Sorescu et al., 2001; Souza et al., 2000; Szocs et al., 2002) and that antioxidants attenuate neointimal hyperplasia (Ghigliotti et al., 2001; Kappert et al., 2006; Nunes et al., 1997; Souza et al., 2000). Furthermore, recent evidence indicates that PDGF itself stimulates reactive oxygen species production in VSMC (Lyle and Griendling, 2006; Sorescu et al., 2001) and that the VSMC response to PDGF is reactive oxygen species-dependent (Sundaresan et al., 1995). These findings strongly suggest that reactive oxygen species may play a central role in VSMC proliferation and migration. Therefore, reduction of reactive oxygen species could be an effective therapeutic intervention in atherosclerosis and restenosis.

The dry roots of *Salvia miltiorrhiza* (Danshen) are a representative oriental medicine used for the treatment of coronary heart disease, cerebrovascular disease, hepatitis, liver cirrhosis, chronic renal failure, dysmenorrhea, and insomnia (Cheng, 2007; Zhou et al., 2005). The chemical constituents of Danshen have been studied since the early 1930s. Early studies focused mainly on lipophilic compounds, such as tanshinones, whereas recent studies have focused more on hydrophilic compounds. Indeed, at least 50 compounds have been isolated and identified from the aqueous extracts of Danshen (Liu et al., 2006; Wu et al., 2000; Zhou et al., 2005). Lithospermic acid B, the tetramer of caffeic acid, is the most abundant component in aqueous extracts of the *Salvia* species and is present mainly as a magnesium salt (Fig. 1). Magnesium lithospermate B is known to have antioxidative (Huang and Zhang, 1992; Wu et al., 2000) and antifibrotic (Jung et al., 2002; Shigematsu et al., 1994) effects. In addition, numerous studies have reported that magnesium lithospermate B has renoprotective (Yokozawa et al., 1997; Yokozawa et al., 1992) and myocardial salvage (Fung et al., 1993) effects. Previously, we reported an effective method for isolating magnesium lithospermate B from Danshen (Jung et al., 2002) and found that magnesium lithospermate B, a potent antioxidant, had a protective effect on diabetes-induced renal disease (Lee et al., 2003).

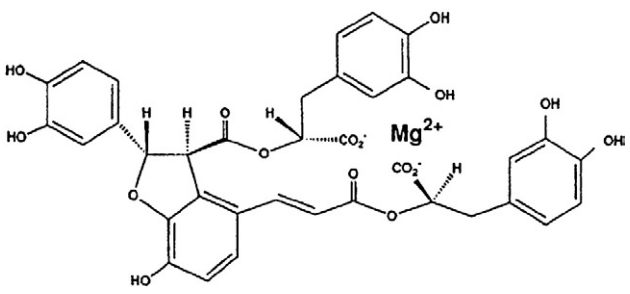

Fig. 1. Chemical structure of magnesium lithospermate B.

Based on the above considerations, we investigated the therapeutic potential of magnesium lithospermate B in injury-induced neointimal hyperplasia in rat carotid arteries as well as its effects on PDGF-induced VSMC proliferation and migration in culture in order to gain insight into the intracellular mechanism(s) of actions of magnesium lithospermate B.

2. Materials and methods

2.1. Materials

Magnesium lithospermate B was isolated from dried *S. miltiorrhiza* roots as previously described (Jung et al., 2002). Chemically PDGF is dimeric glycoprotein composed of A or B chains, which are able to form three isoforms: PDGF-AA, PDGF-BB, and PDGF-AB. In this study, we used recombinant rat PDGF-BB purchased from R&D System. Total Akt, phospho-Akt (Ser473) (p-Akt), total p44/p42 MAPK (ERK 1/2), and phospho-p44/p42 MAPK (Thr202/Tyr204) (p-ERK1/2) antibodies were purchased from Cell Signaling Technology. Proliferating cell nuclear antigen (PCNA) antibody was purchased from Santa Cruz. 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2-tetrazolium bromide (MTT), xanthine, xanthine oxidase, lucigenin, wortmannin, a phospatidylinositol 3-kinase (PI3K) inhibitor, U0126 (1,4-diamino-2,3-dicyano-1, 4-bis[2-aminophenylthio] butadiene), an ERK1/2 inhibitor, and *N*-acetylcysteine were purchased from Sigma.

2.2. VSMC isolation and culture

Rat VSMCs were isolated from the thoracic aorta of Sprague–Dawley rats (250–300 g; ORIENT-Charles River Technology, Seoul, Korea) as described previously (Lee et al., 2006). More than 95% of the cells were positive for α-actin and exhibited the typical hill-and-valley morphology of VSMCs. In this study, cell passages between four and eight were used. VSMCs were grown in Dulbecco-modified Eagle's medium (DMEM) (Sigma Chem, St. Louise, MO) supplemented with 10% fetal bovine serum (FBS) to subconfluence and synchronized by serum-deprivation (0.1% FBS) for 24 h. Synchronized cells were treated with magnesium lithospermate B or other agents for 24 h prior to PDGF-BB stimulation. PDGF-BB (10 ng/ml) was applied to VSMCs for 6 h in the migration assay, 48 h for the MTT assay and evaluation of PCNA expression, and 10 min for analysis of Akt and ERK activation.

2.3. Reactive oxygen species assay

First, the direct reactive oxygen species scavenging effect of magnesium lithospermate B was examined in the test tube without cells. Different concentrations of magnesium lithospermate B

were added to a xanthine/xanthine oxidase mixture (Liu et al., 2006). Xanthine/xanthine oxidase-induced production of reactive oxygen species (mainly superoxide) was measured by lucigenin-enhanced chemiluminescence and monitored for 10 min with a luminometer (Microlumat LB96, Berthold, EG&G). Second, the effect of magnesium lithospermate B on intracellular reactive oxygen species levels was examined in vital cells as described previously (Ohba et al., 1994). Briefly, VSMCs (1×10^5 cells) were seeded on a 35 mm dish in 1 ml of DMEM containing 10% FBS, and synchronized as described above. Synchronized VSMCs were pretreated with different concentrations of magnesium lithospermate B in serum-free medium for 1 h. Cells were then incubated for 15 min at 37 °C with CM-H_2DCF-DA (Molecular Probes Inc., Eugene, OR, USA) in Hank's balanced salt solution (HBSS)-*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) and then stimulated with 10 ng/ml PDGF-BB for 10 min or 100 μM H_2O_2 for 1 min. Lastly, fluorescence intensity was measured by CoolSNAP camera system with BX51 microscopy (Olympus, Japan).

2.4. Magnesium lithospermate B treatment protocol and rat carotid artery balloon injury

Male Sprague–Dawley rats (300–350 g; ORIENT-Charles River Technology, Seoul, Korea) were separated into two groups, either the vehicle treated group ($n=13$) and the magnesium lithospermate B treated group ($n=25$). Vehicle (D.W) or magnesium lithospermate B (10 mg/kg/d) was administered daily via i.p. beginning one day prior to balloon injury and lasting until 28 days after the balloon injury. The balloon injury was performed as previously described (Kappert et al., 2006). Briefly, under inhalative isofluran-anesthesia, the left carotid artery was isolated and a Fogarty 2F embolectomy catheter (Edward life Sciences) was introduced through an external carotid arteriotomy incision, advanced to the aortic arch, inflated to produce moderate resistance, and gradually withdrawn three times (Jagadeesha et al., 2005; Leppanen et al., 2000). This study was performed according to a protocol approved by the Institutional Animal Care and Use Committee of Yonsei University in accordance with the Guide for the Care and Use of Laboratory Animals.

2.5. Tissue processing, staining, and morphometric analyses

Twenty-eight days after balloon injury, the common carotid arteries were excised bilaterally, fixed, and embedded in paraffin (Kappert et al., 2006). Six-μm sections of paraffin-embedded tissue were stained with hematoxylin–eosin. Morphometric analysis was performed from two to four individual sections from the middle of each injured arterial segment. The image of each carotid artery section was analyzed by computerized morphometry (SCN Image). The luminal, intimal, and medial areas were calculated for each arterial cross section. Neointimal formation was expressed as the percentage of intima of media and intima ([intima/media+intima]×100 (%)).

2.6. Cell proliferation analysis

Cell proliferation was assessed by a modified MTT assay as described previously (Yan and Hansson, 1998). VSMCs were grown to subconfluence in 24-well plates and synchronized as described above in the presence or absence of the indicated drugs for 24 h. Under these conditions, cells were stimulated with PDGF-BB for 48 h and washed with phosphate buffer saline (PBS). Next, 300 μl of an MTT solution (0.5 mg/ml) was added to each well and the plates were incubated again for 4 h at 37 °C. Finally, the MTT-containing medium was removed by aspiration and 400 μl of dimethylsulfoxide (DMSO) solution was added. After lysing for 10 min, 100 μl from each well was transferred to a 96-well plate. Absorbance was measured at 570 nm using an enzyme-linked immnunosorbent assay (ELISA) reader (Spectra MAX3 340, Molecular Devices Corp, Sunnyvale CA, USA). To validate MTT assay, trypan blue exclusion was performed in parallel.

2.7. Cell migration analysis

Cell migration was assayed with a modified Boyden chamber method as previously described (Weber et al., 2004). VSMCs were grown to 85% confluence and then made quiescent (0.1% FBS) in the presence or absence of the indicated drugs for 24 h. VSMCs (10^4 cells/ml) were transferred to the upper chamber of a transwell plate on a collagen-coated polycarbonate membrane containing 8-μm pores (Costar). VSMCs were then exposed to PDGF-BB in the lower chamber for 6 h, after which nonmigrated cells were removed from the upper chamber using a cotton swab. The cells remaining in the bottom membrane were fixed with ethanol and stained with trypan blue. The membrane was mounted on a slide and then examined under a microscope. Migration activities were quantified by counting the stained cells from four randomly chosen high-power fields (HPFs, ×400) per membrane.

2.8. SDS-PAGE and immunoblotting

Cells were pretreated with the indicated drugs for 24 h and then stimulated with PDGF-BB (10 mg/ml) for 10 min. Cell lysates were subsequently prepared and subjected to Western blot analysis. Membranes were immunoblotted with the indicated antibodies. Peroxidase-conjugated anti-rabbit and anti-mouse antibodies were used as secondary antibodies (Santa Cruz). Immunoreactive bands were visualized using an enhanced chemifluorescent labeling (ECL) kit (Amersham, Buckingham-shire, UK). Films were scanned and the intensities of immunoreactive bands were quantified with a calibrated densitometer (FUJI, Model: LAS-1000).

2.9. Data analysis

All data were expressed as mean±S.E.M. unless otherwise stated. Statistical significance was determined by one-way ANOVA with a Bonferroni post hoc test for comparison.

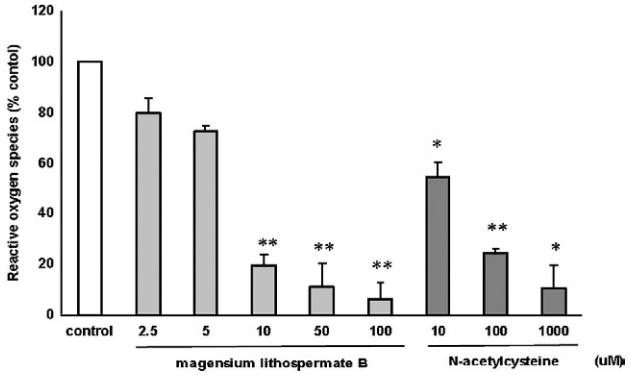

Fig. 2. Reactive oxygen species scavenging effect of magnesium lithospermate B in the xanthine/xanthine oxidase system. Relative fluorescence intensities were measured with a luminometor as described in the Methods section. Results are shown as mean±S.E.M. from five independent experiments in duplicate. *$P<0.05$ and **$P<0.001$ vs. control.

Statistical analyses were performed using the SPSS for Windows software (version 12.0; SPSS, Chicago, IL). A P value of <0.05 was considered statistically significant.

3. Results

3.1. Antioxidant effect of magnesium lithospermate B

Magnesium lithospermate B is known to mediate antioxidant effects, as mentioned above. In this study, we first examined whether the antioxidant effect of magnesium lithospermate B occurs directly by scavenging reactive oxygen species. Reactive oxygen species were generated by xanthine/xanthine oxidase in the test tube, to which magnesium lithospermate B was directly added. As demonstrated in Fig. 2, magnesium lithospermate B decreased the amount of reactive oxygen species in a dose-dependent manner. *N*-acetylcysteine, a well known antioxidant, was tested for comparison. Both agents had a reactive oxygen species scavenging effect; however, the effect of magnesium lithospermate B was more potent than *N*-acetylcysteine (Fig. 2). Next, we tested the antioxidant effect of magnesium lithospermate B in the cultured VSMCs. Magnesium lithospermate B decreased PDGF-BB-induced intracellular reactive oxygen species generation as well as exogenous H_2O_2 in VSMCs (Fig. 3), indicating that the antioxidant effect of magnesium

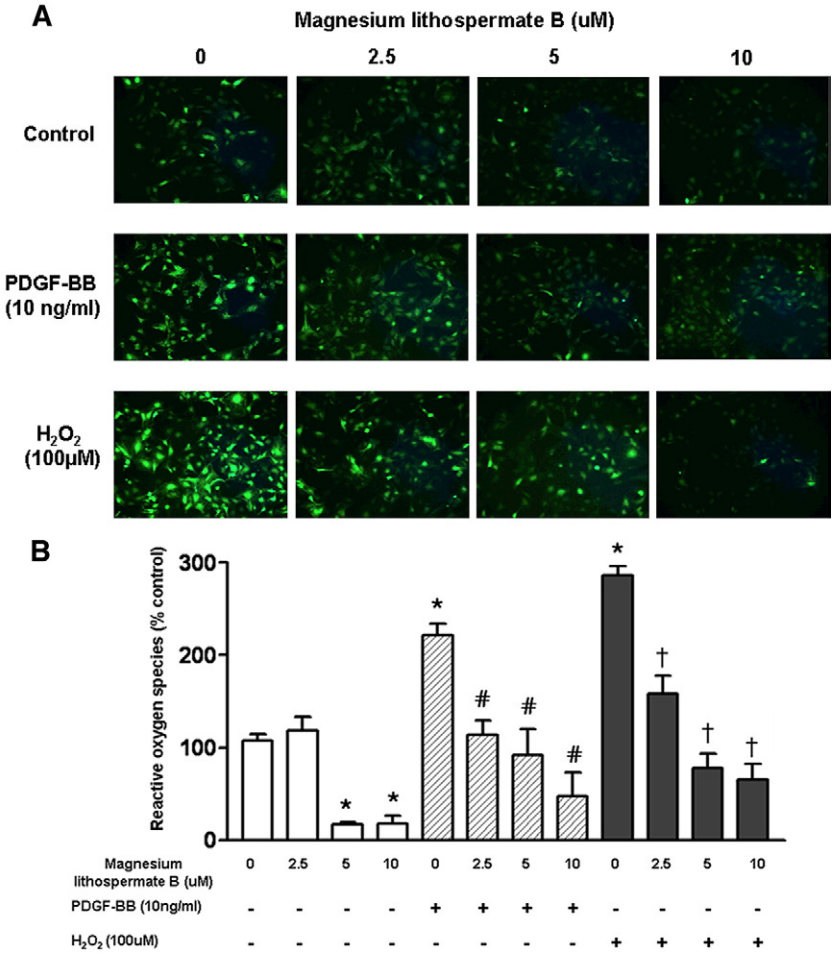

Fig. 3. Antioxidant effect of magnesium lithospermate B in VSMCs. (A) Magnesium lithospermate B diminished PDGF-BB-induced intracellular reactive oxygen species as well as exogenous H_2O_2 in VSMCs. Cells were incubated with PDGF-BB (10 ng/ml) for 10 min or H_2O_2 (100 μM) for 1 min, and reactive oxygen species were assessed as described in the Methods section. (B) Relative fluorescence intensities were calculated using untreated control cells as a standard. Results are shown as mean±S.E.M. from five independent observations. *$P<0.05$, vs. unstimulated control; $^{\#}P<0.05$ vs. PDGF-BB (+) control; $^{\dagger}P<0.05$ vs. H_2O_2 (+) control.

lithospermate B, at least in part, occurs via the direct scavenging of reactive oxygen species (Fig. 3).

3.2. Magnesium lithospermate B inhibits injury-induced neointimal hyperplasia

As reactive oxygen species are known to play an important role in restenosis after angioplasty (Souza et al., 2000; Szocs et al., 2002), we investigated the therapeutic effect of magnesium lithospermate B on injury-induced neointimal formation in rat carotid arteries. Animals were treated with either magnesium lithospermate B (10 mg/kg/d, $n=25$) or vehicle ($n=13$), and neointimal formation was measured 28 days after balloon injury. Fig. 4A–F shows photomicrographs of balloon-injured and perfusion-fixed left carotid artery cross-sections. As compared to the sham operated group (Fig. 4A and D), balloon injury stimulated neointimal hyperplasia (Fig. 4B and E); however, it was significantly prevented by magnesium lithospermate B treatment (Fig. 4C and F). As demonstrated in Fig. 4G, neointimal formation was 52.0±9.1% in the vehicle treated group and 27.0±5.2% in the magnesium lithospermate B treated group ($P<0.05$). The inhibitory effect of magnesium lithospermate B on injury-induced neointimal hyperplasia was approximately 48.1%.

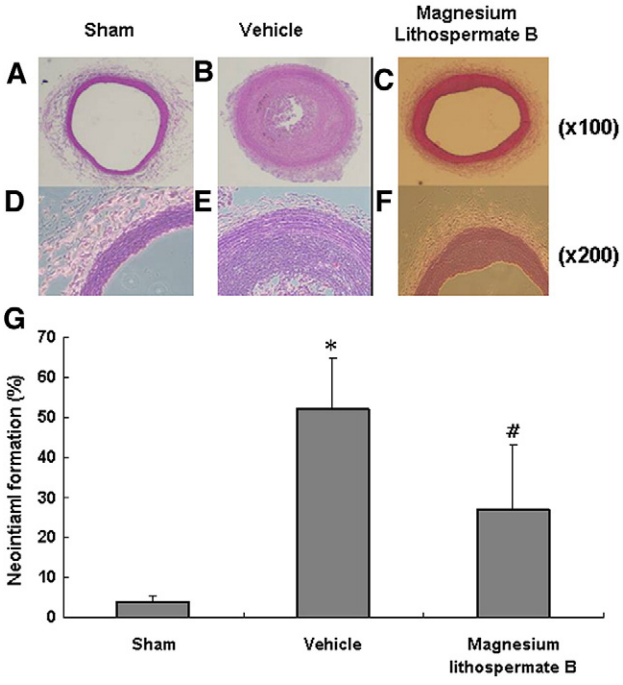

Fig. 4. Effect of magnesium lithospermate B on neointimal formation after carotid artery balloon injury in rats. Rats were subjected to either magnesium lithospermate B (10 mg/kg/d, $n=25$) or vehicle ($n=13$) treatment by i.p. injection beginning one day before injury and lasting until 28 days after the injury. (A–F) Representative histologic sections of neointimal formation. A and D, Sham; B and E, vehicle treatment; C and F, magnesium lithospermate B treatment. (G) Bar graph shows the neointimal formation ([intima/media+intima]×100 (%)). Results are expressed as the mean±S.E.M. *$P<0.05$ vs. sham; $^{\#}P<0.05$ vs. vehicle treatment.

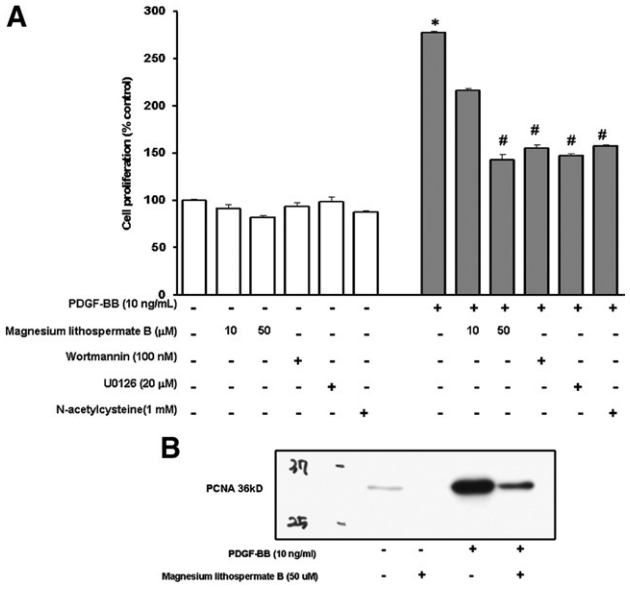

Fig. 5. Effect of magnesium lithospermate B on PDGF-BB-induced VSMC proliferation. (A) Proliferation activities were measured by MTT assay in the absence (left) or presence (right) of PDGF-BB (10 ng/ml). Relative proliferation activities were expressed using untreated control cells as a standard. Results are mean±S.E.M. from five independent experiments performed in triplicate. *$P<0.001$ compared with PDGF-BB (−) control; $^{\#}P<0.001$ compared with PDGF-BB (+) control. (B) Effect of magnesium lithospermate B on PDGF-BB-stimulated PCNA expression in Western blot analysis.

3.3. Magnesium lithospermate B inhibits PDGF-BB-stimulated VSMC proliferation

We tested the effect of magnesium lithospermate B on VSMC proliferation using an MTT assay. When quiescent cells were treated with magnesium lithospermate B (10 or 50 μM) in the absence of PDGF-BB, no significant difference was observed in cell viability (Fig. 5A, left panel), suggesting that magnesium lithospermate B at these two concentrations was not cytotoxic. The lack of cytotoxicity was also ascertained with a trypan blue exclusion assay (data not shown). Stimulation with PDGF-BB increased VSMC proliferation by 2.8-fold (277.4±1.2% in PDGF-BB (+) vs. 100.0±1.2% in the PDGF-BB (−) control, $P<0.001$; Fig. 5A). Magnesium lithospermate B significantly inhibited PDGF-BB-induced VSMC proliferation in a dose-dependent manner, with 49% inhibition observed at 50 μM of magnesium lithospermate B (143.0±5.4% in PDGF-BB (+) treated with magnesium lithospermate B vs. 277.4±1.2% in PDGF-BB (+) control, $P<0.001$; Fig. 5A). *N*-acetylcysteine also significantly inhibited PDGF-BB-induced VSMC proliferation with 43% inhibition (157.4±1.4% in PDGF-BB (+) treated with *N*-acetylcysteine vs. 277.4±1.2% in PDGF-BB (+) control, $P<0.001$; Fig. 5A). These results suggested that magnesium lithospermate B likely inhibited VSMC proliferation by reducing PDGF-BB-induced intracellular reactive oxygen species generation. In parallel with the result of the MTT assay, magnesium lithospermate B markedly reduced PDGF-BB-induced PCNA expression in VSMCs (Fig. 5B).

3.4. Magnesium lithospermate B inhibits PDGF-BB-stimulated VSMC migration

Next, we tested the effects of magnesium lithospermate B on VSMC migration. Fig. 6 demonstrated the results of transwell migration assay representing chemotaxis. PDGF-BB enhanced the basal migration of VSMCs by 2.5-fold (62.0±3.0 cells/HFP after PDGF-BB stimulation vs. 24.7±2.5 cells/HPF in basal migration, $P<0.001$). Treatment with magnesium lithospermate B (10 and 50 μM) resulted in a concentration-dependent inhibition of cell migration with statistical significance achieved at 10 μM (31.0±2.0 cells/HPF; $P<0.001$) and a virtually complete inhibition of cell migration at 50 μM (16.0±1.0 cells/HPF; $P<0.001$). *N*-acetylcysteine also significantly inhibited VSMC migration (17.3±1.5 cells/HPF; $P<0.001$). These data indicated that magnesium lithospermate B inhibited VSMC migration by reducing PDGF-BB-induced intracellular reactive oxygen species generation.

3.5. Magnesium lithospermate B inhibits PDGF-stimulated Akt and ERK1/2 pathways

It is well known that PI3K/Akt and MAPK/ERK are the two main PDGF signaling pathways and are linked to numerous cellular processes, including proliferation and migration (Gennaro et al., 2004; Higaki et al., 1996; Lai et al., 1996). Consistent with previous reports, both the Akt inhibitor wortmannin (100 nM), the ERK inhibitor U0126 (20 μM) reduced PDGF-BB-induced VSMC proliferation (Fig. 5A) and migration (Fig. 6). Thus, we investigated the effects of magnesium lithospermate B on these signaling pathways in VSMCs. PDGF-BB stimulated the phosphorylations of downstream effectors, Akt (Fig. 7A) and ERK1/2 (Fig. 7B); however, these phosphorylations events were blocked by magnesium lithospermate B (Fig. 7A and B). *N*-acetylcysteine also inhibited the PDGF-BB-stimulated phosphorylation of Akt (Fig. 7A) and ERK1/2 (Fig. 7B). Together, these results suggest that magnesium lithospermate B inhibited the upstream signaling cascade of PI3K/Akt and MAPK/ERK, perhaps by reducing PDGF-BB-induced intracellular reactive oxygen species generation.

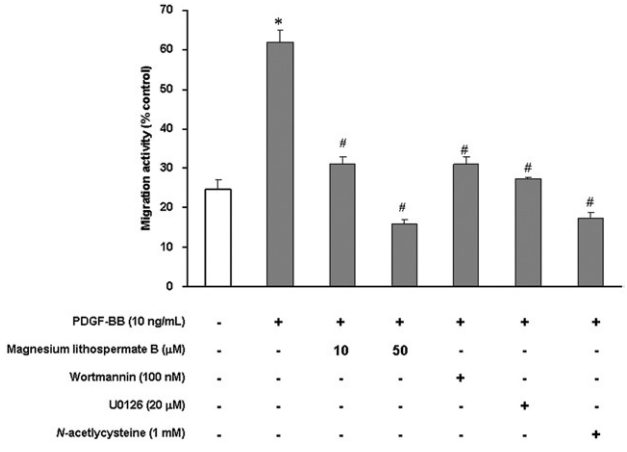

Fig. 6. Effect of magnesium lithospermate B on PDGF-BB-induced VSMC migration. VSMCs were stimulated with 10 ng/ml PDGF-BB for 6 h. Migration activities were quantified as the mean numbers of migrated cells observed in 4 HPFs. Results are mean±S.E.M. from five independent experiments performed in triplicate. *$P<0.001$ vs. PDGF-BB (−) control; #$P<0.001$ vs. PDGF-BB (+) control.

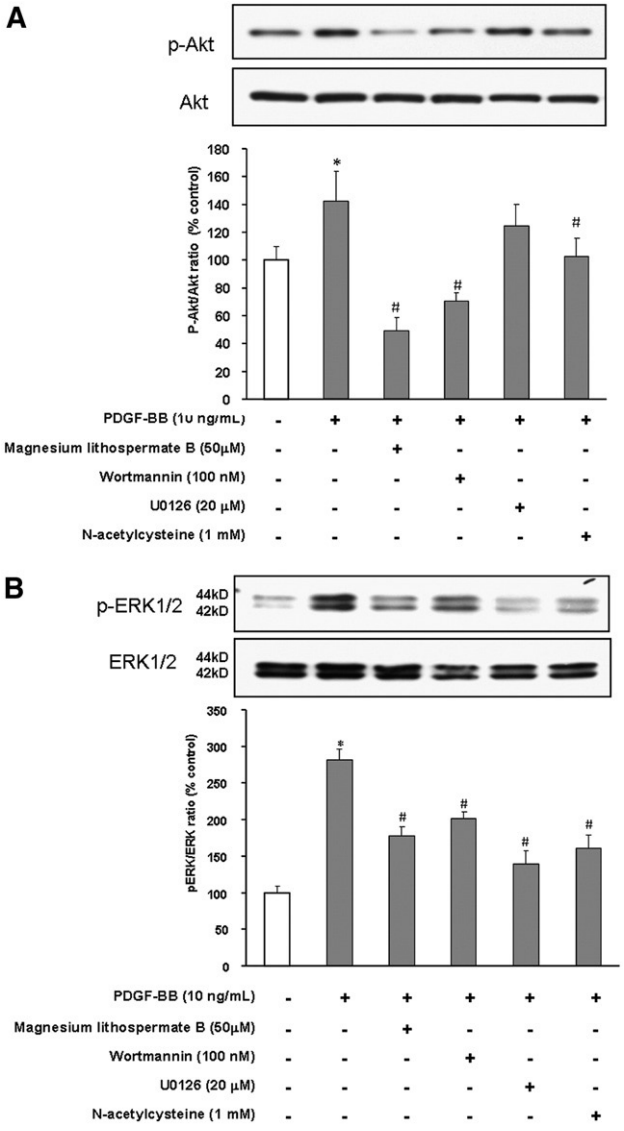

Fig. 7. Effects of magnesium lithospermate B on Akt and ERK signaling pathways. (A) p-Akt and Akt. (B) p-ERK1/2 and ERK1/2. VSMC were serum-starved for 24 h in the absence or presence of the indicated drugs and stimulated with PDGF-BB for 10 min. Pharmacological inhibitors of Akt (wortmannin 100 nM) and ERK1/2 (U0126 20 μM) were treated. Whole cell lysates were prepared and subsequently used for detection of p-Akt, Akt, p-ERK1/2, or ERK1/2 by Western blot. The bar graph shows the ratio of densities of phosphoproteins to total proteins for the blots. Data are expressed as mean±S.E.M from three independent experiments. *$P<0.05$ vs. PDGF-BB (−) control; #$P<0.05$ vs. PDGF-BB (+) control.

4. Discussion

We report here that magnesium lithospermate B, a potent reactive oxygen species scavenger, significantly inhibited injury-induced neointimal formation and PDGF-BB-induced

VSMC proliferation and migration. Magnesium lithospermate B inhibited both the PDGF-BB-stimulated PI3K/Akt and MAPK/ERK signaling pathways, suggesting that the antioxidant effect of magnesium lithospermate B was at least in part attributable to the inhibition of PDGF signal transduction.

Neointima-induced vessel stenosis after percutaneous angioplasty is a major clinical complication, occurring in 30% to 50% of patients within three to six months of the procedure (Detre et al., 1988). In this study, a well-established carotid injury model was used to investigate the ability of magnesium lithospermate B to protect against neointimal formation. Our *in vivo* findings strongly suggested that magnesium lithospermate B was capable of protecting against injury-induced pathological remodeling of blood vessels.

In this study, magnesium lithospermate B inhibited PDGF-BB-stimulated Akt as well as ERK1/2 phosphorylations. This result suggested that magnesium lithospermate B may inhibit PDGF signaling by acting upstream of Akt and ERK1/2. PDGF is known to regulate the tyrosine phosphorylation of a variety of signaling proteins via intracellular production of H_2O_2 (Bae et al., 2000; Heldin and Westermark, 1999; Sundaresan et al., 1995). The level of tyrosine phosphorylation during growth signaling is determined by the relative activity of tyrosine kinase and protein tyrosine phosphatase, and a large series of studies suggest that H_2O_2-mediated receptor tyrosine kinases activation involves the inactivation of protein tyrosine phosphatases (Rhee et al., 2005). PDGF β-receptors and other tyrosine kinases involved in VSMC proliferation and migration are regulated by protein tyrosine phosphatase. Recently, Kappert et al. reported that *N*-acetylcysteine treatment prevented H_2O_2-induced protein tyrosine phosphatase inhibition and reduced H_2O_2-induced PDGF β-receptor phosphorylation, PDGF-induced proliferation, and chemotaxis of VSMCs (Kappert et al., 2006). Although we did not investigate the effect of magnesium lithospermate B on PDGF β-receptor phosphorylation and protein tyrosine phosphatase activity in this study, our data strongly support the hypothesis that magnesium lithospermate B may increase protein tyrosine phosphatase activity by scavenging PDGF-induced reactive oxygen species, thereby blocking PDGF signaling cascade such as PI3K and MAPK, which are responsible for VSMC proliferation and migration. Nevertheless, VSMCs are unlikely to be regulated solely by PDGF during neointimal hyperplasia after injury. Our *in vivo* findings suggest that magnesium lithospermate B may block signaling pathways of not only PDGF, but other growth factors or cytokines as well.

As mentioned in the Introduction, magnesium lithospermate B is a naturally occurring compound in Danshen. This herb has traditionally been used to treat vascular disease for centuries and no serious adverse effects of Danshen have been reported so far. Although better-designed studies are essential to prove or refute the existence of serious adverse events caused by Danshen products, wide clinical usage over many years in China appears to indicate that Danshen products are safe (Zhou et al., 2005; Cheng, 2007).

In conclusion, our findings suggest that magnesium lithospermate B is capable of preventing injury-induced neointimal hyperplasia. We believe this beneficial effect of magnesium lithospermate B may be due to a reactive oxygen species scavenging effect that attenuates the signaling cascades of PDGF as well as other growth factors and cytokines. Our results provide novel insights into the protective action of magnesium lithospermate B on the vascular injury response, suggesting a potential therapeutic application of magnesium lithospermate B in atherosclerosis and restenosis after angioplasty.

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