Lithospermate-B attenuates PDGF-BB-induced proliferation of vascular smooth muscle cell

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Hye Jun Seo

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2004. 저자 씀

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Abstract

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Radix Salviae Miltiorrhizae, a traditional Chinese herb known as “Danshen”, has been used in clinics to improve blood circulation. However, a precise mechanism for its biologic function has not been clearly represented yet. Platelet-derived growth factor-β (PDGF-BB) receptor stimulates vascular smooth muscle cell (VSMC) hypertrophy, which is a critical event in the development of

atherosclerosis and restenosis after angioplasty. It has been reported that PDGF-BB induced reactive oxygen species (ROS), which mediate numbers of intracellular signaling responsible for VSMC hypertrophy.

Lithospermate-B (LAB), a main active component of Danshen, is known as a potent ROS scavenger. Therefore, we investigated the inhibitory effects of LAB on PDGF-BB-induced VSMC proliferation. We demonstrated that 1) LAB inhibited PDGF-BB-induced proliferation of VSMC in a dose-dependent manner; 2) treatment with PDGF-BB induced increase of the intracellular ROS levels, while inhibition of ROS production by pretreatment with NAC or LAB suppressed VSMC growth; 3) Akt was phosphorylated and activated upon PDGF-BB treatment in a ROS-dependent manner; and 4) ERK was also activated by PDGF-BB treatment, while pretreatment with NAC or LAB suppressed its activation.

These results collectively, indicate that LAB suppresses PDGF-BB-induced ROS production and subsequent proliferation of VSMCs by inhibition of Akt and ERK pathways. Therefore, we propose that LAB may be used as a new therapeutic agent for the treatment of atherosclerosis.

Key Words : atherosclerosis, Lithospermate-B, PDGF-BB, ROS, antioxidant.

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I. Introduction

Atherosclerosis is a principal cause of myocardial infarction, stroke and peripheral vascular diseases and accounts for nearly half of all mortality in developed countries. For example, it has been estimated that atherosclerosis leads to approximately 500,000 deaths from coronary artery diseases and 150,000 deaths from stroke every year in the United States (American Heart Association, 1996)[1]. Vascular smooth muscle cells (VSMCs) are one of the major constituents

of blood vessel walls responsible for the maintenance of vessel structures and physiologic functions [2]. Hypertrophy, hyperplasia and migration of the VSMCs are considered to be key events in the development of atherosclerosis [1,3,4,5,6,7,8,9].

Various stimuli such as growth factors, oxidative stresses, and mechanical stress are implicated in these processes. Platelet-derived growth factor (PDGF), a disulfied-bond-linked dimmer, binds to a protein tyrosine kinase receptor that regulates many cellular functions including gene expression, proliferation, differentiation and cell mobility. Hypertrophic effect of PDGF-BB has been well recognized in various cell types, especially in VSMCs. Therefore, inhibition of PDGF-BB-induced VSMCs hypertrophy prevents the development of cardiovascular diseases [10]. In addition, stimulation of VSMCs by PDGF-BB increases production of intracellular reactive oxygen species (ROS) [11].

ROS are very unstable and highly reactive and tend to initiate chain reactions that result in irreversible chemical changes in macromolecules such as proteins or lipids. These deleterious reactions can result in cellular dysfunction and death [12]. For many years, ROS such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) have been thought as toxic by-products of aerobic cellular respiration. Recently, a variety of evidence suggests that ROS are involved in the signal transduction in mammalian cells [13].

Numerous studies have shown that ROS and growth factors such as PDGF-

BB activate multiple signaling pathways including the Raf–MEK1–MAPK and phosphatidyl inositol 4,5-bisphosphate 3-kinase (PI3K)-Akt pathways. The p42/44 extracellular-regulated kinase (ERK 1/2), a member of the MAPK family, is a well-characterized cellular signaling molecule in VSMCs [14,15]. Its activation involves multiple membranous and cytosolic proteins in a cascade of phosphorylation events that ultimately lead to regulation of gene transcription. Sequential phosphorylation cascade includes activation of Raf, MAPK kinase 1/2 (MEK-1/2) and finally ERK 1/2. Activation of ERK 1/2 is a central component of growth factor signal transduction and has been shown to be essential for proliferation and migration of VSMCs. In addition, several studies have shown that PDGF-BB and ROS may also promote proliferation of VSMCs by activating another protein kinase pathway that is linked to cell survival, the PI3K and Akt (also called as protein kinase B or PKB) pathway.

Many studies have indicated that the MAPK and Akt pathways lead to two distinct end effectors and are regulated independently by various stimulators and intermediate signal transduction molecules. Recent studies, however, have suggested that these two pathways also cross-talk possibly via interaction between Raf and Akt [16, 17, 18].

Radix salviae miltiorrhizae, a traditional Chinese medical herb known as "Danshen", has been wildely use in clinics to improve blood circulation, relieve

blood stasis and eliminate swelling. In addition, it has been reported to have vasodilator, hypertensive, anticoagulam, and antibacterial activities and have a beneficial effect in rats with chronic renal failure. Lithospermate B (LAB) Lithospermate B (LAB) was recently isolated from *S. miltiorrhizae* and was found to have significant beneficial effects on renal injury of diabetic rats, possibly by reducing ROS [20, 21, 22, 23].

Thus, our present study has examined the effect of LAB on VSMCs proliferation to evaluate the role of LAB's ROS scavenging effects under the influence of PDGF-BB.

II. Materials and Methods

1. Rat VSMCs isolation and culture

Thoracic aorta from 6- to 8-week-old male Sprague-Dawley rats were removed and transferred on ice into serum-free Dulbecco's modified Eagle's medium (DMEM) containing 100 units/ml of penicillin and 100 μg/ml of streptomycin. The aorta was freed from any connective tissues, and transferred into a Petri dish containing 5 ml of DMEM with 1 mg/ml of collagenase type I (Sigma-Aldrich, St. Louis, MO, USA) and 0.5 mg/ml elastase (Upstate Biotechnology, Cleveland, OH, USA). The aorta was incubated for 30 min at 37°C and then transferred into DMEM, and the adventitia was stripped off with forceps under a binocular microscope. The aorta was next transferred into a plastic tube containing 5 ml of the enzyme dissociation mixture described above and incubated for an additional 2 h at 37°C. Suspension was centrifuged (1,500 rpm for 10 min) and the pellet was resuspended in DMEM with 10% fetal bovine serum (FBS). Resulting cells were cultured over several passages (up to 10) for following experiments. Rat VSMCs were cultured in DMEM supplemented with 10% FBS, 1% Penicillin - streptomycin mixture in a 10 cm^2 dish at 37°C in a humidified atmosphere of 95% air and 5% CO_2 . Subcultures were prepared from

confluent cultures by 0.25% trypsin-EDTA. These VSMCs were then grown in DMEM containing 10% FBS to near confluence (80%) and incubated further in serum-free medium for 24 h to arrest and synchonize cell growth. The medium was then changed to serum-free DMEM containing different concentrations of LAB as well as the indicated reagents in the presence or absence of platelet-derived growth factor (PDGF-BB) (Sigma- Aldrich, St. Louis, MO, USA). Treatment of the cells with LAB up to 30 μg/ml did not show any recognizable cytotoxicity.

2. Cell proliferation assay

A. LDH assay

VSMCs (1×10^4 cells) were seeded on 12 well plates in 400 μl of DMEM containing 10% FBS, and synchonization was achieved by serum starvation for 24 h. VSMCs were pretreated in serum-free medium in the absence or presence of 1,5,10,20 and 30 μM of LAB, 10 μM U0126, 40 μM LY294002 and 20 mM NAC for 1 h. Then, cells were treated with PDGF-BB (10 ng/ml). After 24 h, the cells were washed in PBS twice and lysed using for 0.5% Triton-X100. Enzymatic activity of lactate dehydrogenase (LDH) was measured using a CytoTox-ONE™

Assay Kit (Promega, Madison, WI, USA). 96-well assay plates containing 50μl of cell lysate were set up. A volume of CytoTox-ONE™ reagent equal to the volume of cell lysate was added and mixed or shaken for 30 seconds. Stop Solution (25μl) was added to each well in the 96-well format. Plates were shaken for 10 seconds and then the fluorescence was recorded at 560/590nm. Total number of live cells was directly proportional to the background-subtracted fluorescence values, which represented the LDH activity.

B. MTT assay

VSMCs (1×10^4 cells) were seeded on 12 well plates in 400 μl of DMEM containing 10% FBS, and synchonization was achieved by serum starvation for 24 h. VSMCs were pretreated in serum-free medium in the absence or presence of 1,5,10,20 and 30 μM of LAB, 10 μM U0126 [1,4-diamino-2,3-dyacyano-1,4-bis(2-aminophenylthio)butandiene; a MEK inhibitor], 40 μM LY294002 [2-(4-morpholinyl)-8-phenyl]-4*H*-1-benzopyran-4-one; a PI3K inhibitor] and 20 mM N-acetylcysteine (NAC) for 1 h. Then, cells were treated with PDGF-BB (10 ng/ml). After 24 h, number of cells was determined. Proliferation of cultured cells was measured by using a colorimetric method based on the metabolic reduction of soluble yellow methylthiazoletetrazolium (MTT) (Sigma-Aldrich, St.Louis, MO,

USA) to its insoluble blue formazan by the action of mitochondrial succinyl dehydrogenase. For measurement of VSMC proliferation, 0.5 μg/ml of MTT was added and incubated for 4 h. After this, 400 μl of dimethylsulfoxide (DMSO) was added to dissolve the formazan crystals that had been formed. Optical density was measured with an ELISA plate reader (Spectra MAX3 340) (Molecular devices Corp, Sunnyvale CA, USA) using a test and reference wavelength of 570nm.

3. Measurement of intracellular ROS levels

VSMCs (1×10^5 cells) were seeded on a 35mm dish in 1 ml of DMEM containing 10% FBS, and synchonization was achieved by serum starvation for 24 h. VSMCs were pretreated in serum-free medium by 1, 5, 10, 20 and 30 μM of LAB for 1 h. Then they were stimulated with 10 ng/ml PDGF-BB or 10 μM H_2O_2 1min, and incubated in the dark for 10 min in 5-(and-6)-chloromethyl-2',7'-dichlorohydorofluorescein diacetate (CM-H_2DCF-DA: Molecular Probes Inc., Eurogene, OR, USA). A fluorescence microscope imaging system was used to inspect culture dishes and ROS generation was visualized.

Intracellular ROS levels were measured as described previously [24]. In brief, cells in a 35 mm dish were incubated with PDGF-BB for10 min and H_2O_2 for 1 min. Then cells were washed with Hanks Balanced Salt Solution (HBSS) (Jeil

Biotechservices Inc, Daegu, South Korea) and incubated in the dark for 10 min in HBSS containing 2.5 mM of DCF-DA. DCF-DA is a nonpolar compound that readily diffuses into cells, where it is hydrolyzed to the nonfluorescent polar derivative 2'7'-dichlorofluorescin (DCF). Culture dishes were transferred to a Leica DM IRB/E inverted microscope, equipped with a 20x Fluotar objective and a Leica TCS NT confocal attachment, and ROS cellular fluorescence was detected (excitation, 488 nm; emission, 515 to 540 nm). The effect of DCFH photooxidation was minimized by collecting the fluorescence image with a single rapid scan, and identical parameters, such as contrast and brightness, were used for all determinants.

4. Western blot analysis

VSMCs (2×10^5 cells) were seeded on a 60 mm dish in 3 ml of DMEM containing 10% FBS, and synchonization was achieved by serum starvation for 24 h. For determination of optimal time point for Akt and ERK 1/2 posphorylation, VSMCs were treated with 10 ng/ml of PDGF-BB for varying time periods ranging from 5 min to 1 h. Also, VSMCs were pretreated in serum-free medium by 1,5,10,20 and 30 μM of LAB, 10 μM U0126, 40 μM LY294002 and 20 mM NAC for 1 h. Then cells were stimulated with PDGF-BB (10 ng/ml)

for an additional 10 min.

Then, cells were rapidly washed with ice-cold PBS and lysed in a lysis buffer (25 mM Tris·HCl, pH 7.4, 25 mM NaCl, 25 mM NaF, 25 mM sodium pyrophosphate, 1 mM sodium vanadate, 2.5 mM EDTA, 2.5 mM EGTA, 0.05% (wt/vol) Triton X-100, 0.5% (wt/vol) SDS, 0.5% (wt/vol) deoxycholate, 0.5% (wt/vol) Nonidet P-40, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 mM PMSF). Cells were scraped off and collected, centrifugated at 13,000 rpm for 15 min, and the supernatant was measured for protein concentration by the Bradford assay. Proteins were denatured and subjected to SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated for 3 h at room temperature with 5% (wt/vol) non-fat dry milk in TBS with 0.1% Tween 20 (TBS-T) added. Then, the blots were incubated for 16 h at 4℃ with anti-ERK 1/2 or Akt antibodies. (Cell signaling Technology, Beverly. MA, USA), The membranes were washed with TBS-T four times and incubated with a 1:2000 dilution of an anti-rabbit IgG antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Delaware Avenue, CA, USA.) for 1 h at room temperature. The immunoreactive bands detected by ECL reagents were developed by Hyperfilm-ECL (Amersham Life Science, Little Chalfont, UK). Positive immunoreactive bands were quantified densitometrically and compared to the control.

III. Results

1. Effect of LAB and various pharmacological inhibitors on PDGF-BB-induced VSMC proliferation

We examined the possibility that LAB may inhibit PDGF-BB-induced VSMC proliferation. Cells were made quiescent by serum starvation for 24 h and were treated with different concentrations of LAB (1, 5, 10 and 20 μM) or vehicle for 1 h followed by stimulation with 10 ng/ml PDGF-BB for an additional 24 h. We utilized LDH and MTT assay to determine the effect of LAB on PDGF-BB-induced proliferation of VSMC (Fig. 1). Treatment with PDGF-BB-induced proliferation of VSMC (~70%), while preincubation with LAB suppressed PDGF-BB-induced proliferation in a dose-dependent manner (Fig. 1A). Furthermore, treatment of LAB up to 30μM did not show any discernable cytotoxicity (data not shown) as estimated by the MTT and LDH assay, and this means that its antiproliferative action was not due to a cytotoxic effect. Similar results were obtained by the cell counting assay (data not shown).

To delineate the molecular mechanism by which LAB mediates the inhibitory effect of PDGF-BB-induced VSMC proliferation, we assessed the

effect of the several signal pathway inhibitor, a MEK inhibitor U0126 (20 μM) and a PI3K inhibitor LY294002 (40 μM), and the intracellular antioxidant NAC (20 mM) on the PDGF-BB stimulated VSMC. Treatment with U0126, LY294002 and NAC alone had no significant effect on VSMC viability. However, U0126, LY294002 and NAC significantly inhibited the PDGF-BB-induced proliferation (Fig. 1B).

On the contrary, a p38 MAPK inhibitor SB202190 (10 μM) and a JNK inhibitor JNK inhibitor I (10 μM) have no effect on PDGF-BB-induced proliferation in VSMC (data not shown).

2. Effects of LAB on PDGF-BB induced intracellular ROS in VSMC

A number of recent studies have suggested that PDGF stimulates ROS production in VSMCs [11, 25, 26]. By measuring the intracellular generation of ROS with a peroxide-sensitive fluorophore DCF, we examined whether ROS could be generated in VSMCs by PDGF-BB stimulation. The release of ROS, which was captured as the fluorescence generated within the cells using confocal microscopy, revealed that exposure of quiescent VSMCS to PDGF-BB (10 ng/ml) resulted in a rapid increase in DCF fluorescence by 2.7-fold (Fig. 2B-1). The increase in DCF fluorescence by PDGF-BB was transient. The fluorescence appeared quickly within 5 min and sustained until 20 min. Thus, PDGF-BB was treated for 10 min for the following experiments in this study. We then tested the effect of LAB on PDGF-BB-induced intracellular ROS levels in VSMCs. Rat VSMCs have relatively high levels of DCF fluorescence, while was suppressed by pretreatment with LAB (Fig. 2A). PDGF-BB-induced ROS was significantly inhibited by the pretreatment with LAB in a dose-dependent manner (Fig. 2B). H_2O_2, as a ROS, significantly increased the intracellular ROS compared to the control and this was also inhibited by, the pretreatment with LAB in a dose-dependent manner (Fig. 2C).

3. Effect of LAB and various pharmacological inhibitors on PI3K and ERK 1/2 in the PDGF-BB proliferation

The exact signaling mechanisms leading to VSMC hypertrophy are only partly understood so far. However, it is known that phosphorylation and dephosphorylation of protein kinases play an important role in regulating overall protein synthesis [27].

As indicated in Fig.1B, both U0126 (20 μM), an inhibitor of MEK and LY294002 (40 μM), an inhibitor of PI3K inhibited PDGF-BB-stimulated LDH and MTT activity. To elucidate the signaling events involved in PDGF-BB-induced proliferation, we investigated the activation of ERK 1/2 and Akt (the PI3K downstream kinase) by PDGF-BB in rat VSMCs. Cells were made quiescent by serum starvation for 24 h and treated with PDGF-BB (10 ng/ml) for 5, 10, 20, 30, 60 min. ERK 1/2 and Akt were investigated by immunoblotting with phospho-specific ERK 1/2 and Akt antibodies. Rapid activation of ERK 1/2 and Akt were appeared within 5 min and reaching a maximum after 10 min on Akt (7-fold over unstimulated cells) or 20 min on ERK 1/2 (11-fold over unstimulated cells) (Fig. 3A, 4A). Thus, for following experiments, cells were stimulated with 10 ng/ml PDGF-BB for 10 min.

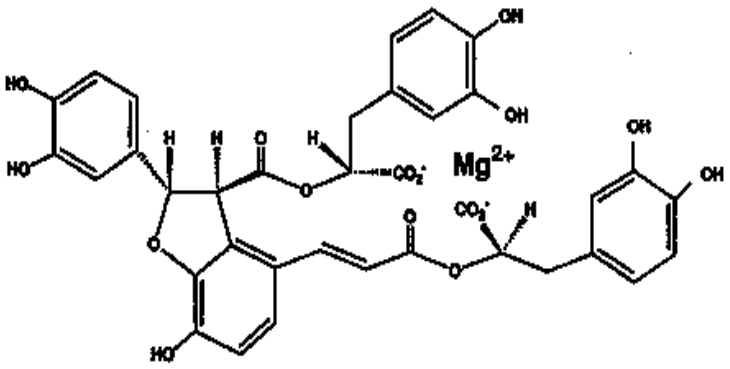
To gain insight about the mechanism of action of LAB, we next examined

whether LAB affects these protein kinase signaling pathways. Cells were made quiescent by serum starvation for 24 h and pretreated with LAB for 1 h. Then cells were treated with 10 ng/ml PDGF-BB for 10 min. As indicated in Fig. 3B and 4B, PDGF-BB-induced Akt and ERK 1/2 phosphorylation was inhibited by the pretreatment with in a dose-dependent manner. Inhibition of PDGF-BB-induced Akt and ERK 1/2 phosphorylation was less prominent at 1, 5, 10 and 20 μM LAB but evident at 30 μM of LAB (1.2-fold inhibition in Akt and 0.7-fold inhibition in ERK 1/2) (Fig. 3B, 4B).

To determine whether effect of LAB in response PDGF-BB is mediated by ROS, VSMCs were pretreated with inhibitors of ROS and LAB before PDGF-BB stimulation. The 11-fold increase in Akt and 2.5-fold increase ERK 1/2 phosphorylation after stimulation with PDGF-BB were inhibited after pretreatment with 30 μM LAB and 20 mM NAC (8-fold inhibition by LAB, 3.4-fold inhibition by NAC in Akt phosphorylation and 0.8-fold inhibition by LAB, 0.6-fold inhibition by NAC in ERK 1/2 phosphorylation) (Fig. 3C, 4C).

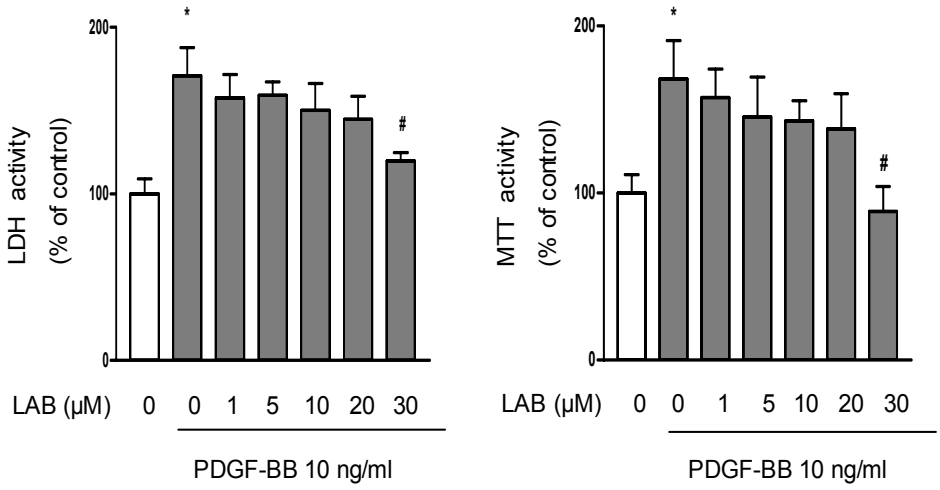
To delineate whether PDGF-BB-induced Akt phosphorylation is downstream or upstream of ERK 1/2 phosphorylation in rat VSMCs, we examined whether U0126 and LY294002 are able to inhibit Akt and ERK 1/2 phosphorylation respectively. Western blot analysis clearly showed that Akt phosphorylation was reduced by only 40 μM LY294002 (3.5-fold inhibition), on the other hand, ERK

1/2 phosphorylation was reduced by both 40 μM LY294002 and U0126 10 μM. These finding indicate that Akt phosphorylation by PDGF-BB may be upstream of ERK 1/2 phosphorylation. (Fig 3C, 4C)



Tabel 1. Structure of Lithospermate-B (MW=717)

A.


LDH activity
(% of control)
200
100
0
*
#
LAB (μM) 0 0 1 5 10 20 30
PDGF-BB 10 ng/ml
MTT activity
(% of control)
200
100
0
*
#
LAB (μM) 0 0 1 5 10 20 30
PDGF-BB 10 ng/ml

B.

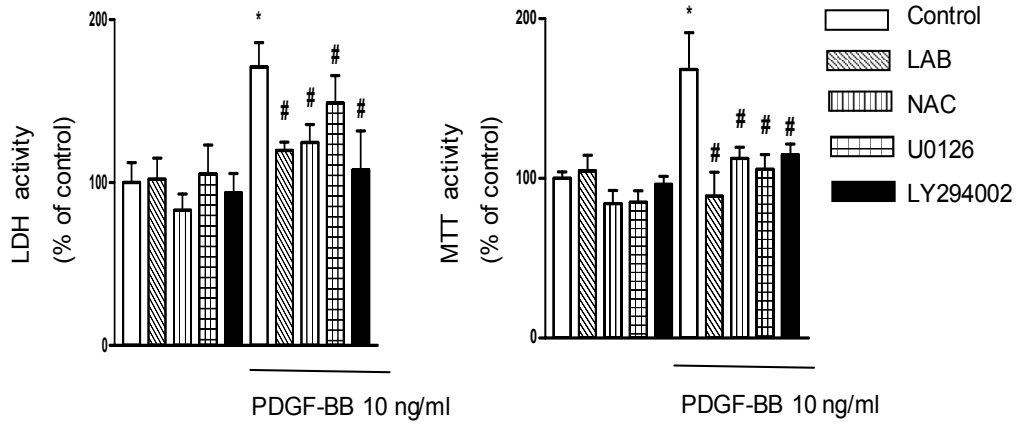
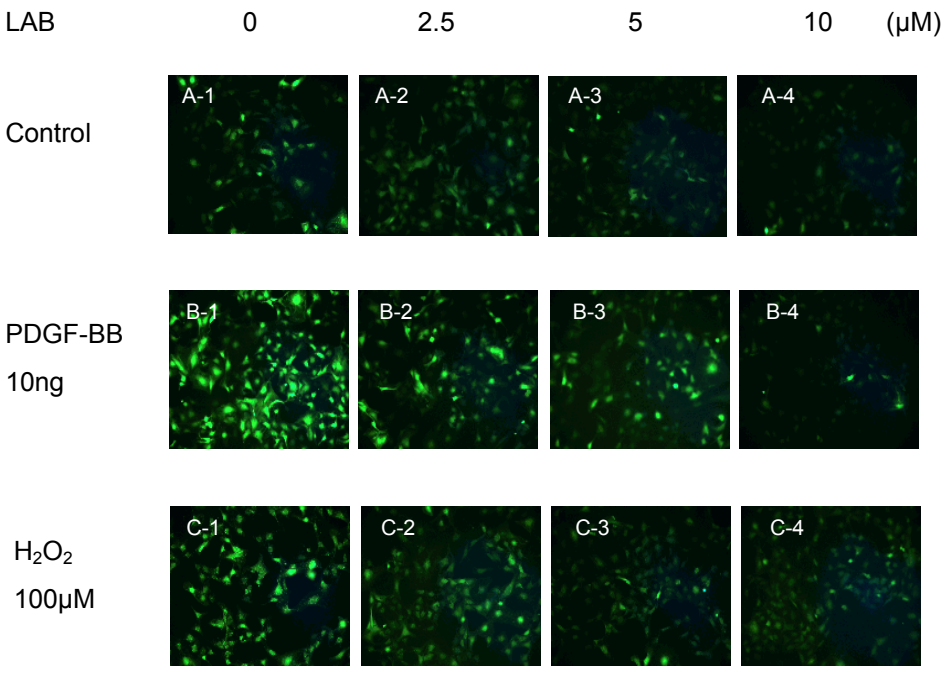

LDH activity
(% of control)
200
100
0
*
#
PDGF-BB 10 ng/ml
MTT activity
(% of control)
200
100
0
*
#
PDGF-BB 10 ng/ml
Control
LAB
NAC
U0126
LY294002

Figure 1. Effects of LAB and various pharmacological inhibitors on PDGF-BB induced proliferation in rat VSMCs. A, VSMCs were incubated in the absence or presence of varying doses of LAB for 1 h and stimulated with 10 ng/ml of PDGF-BB for an additional 24 h. B, VSMCs were incubated in the absence or presence of 10 μM U0126, 40 μM LY294002 and 20 mM N-acetylcysteine (NAC) for 1 h and stimulated with 10 ng/ml PDGF-BB for an additional 24 h. After then, the number of live cells was assessed using LDH and MTT assay. Values are means ± SD. *, $p < 0.001$ compared with the control; #, $p < 0.001$ compared with the presence of PDGF-BB alone treated group (n=18).


LAB
0
2.5
5
10
(μM)
Control
A-1
A-2
A-3
A-4
PDGF-BB
10ng
B-1
B-2
B-3
B-4
H2O2
100μM
C-1
C-2
C-3
C-4

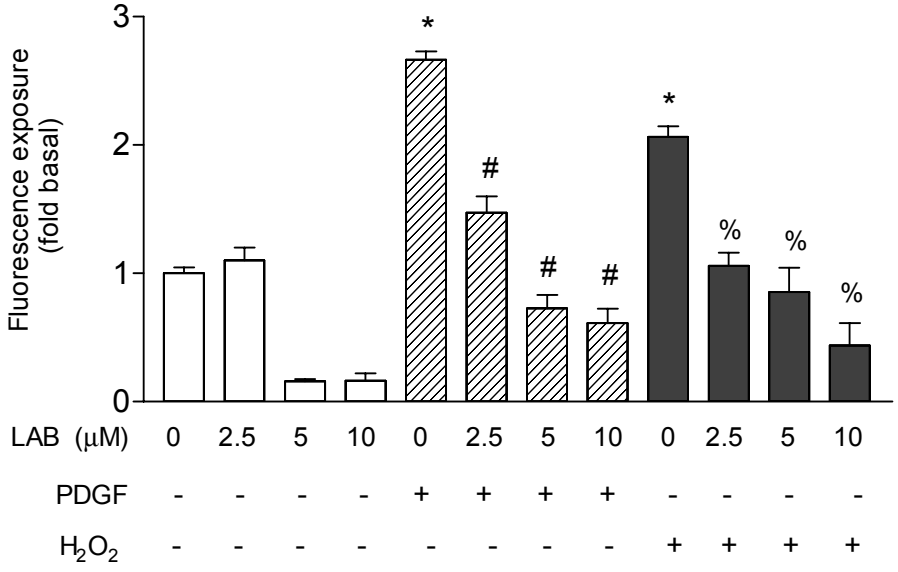
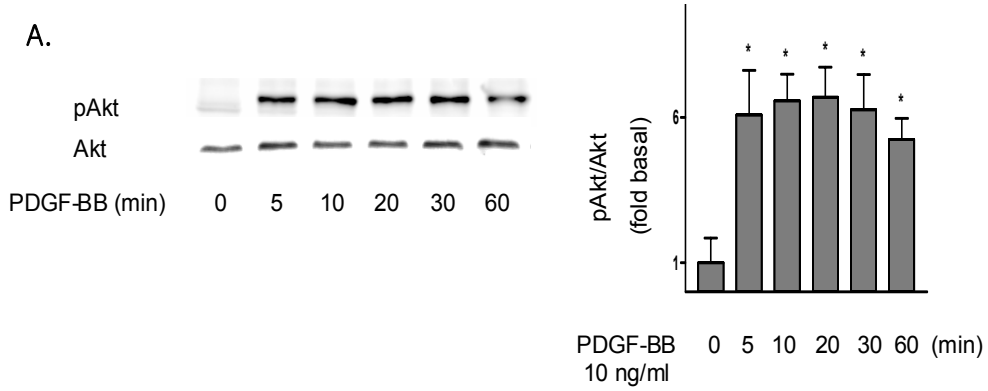
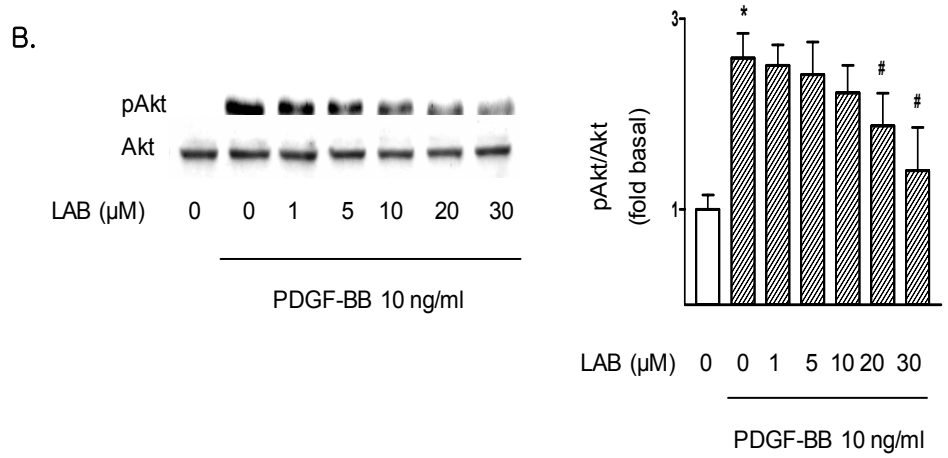

Fluorescence exposure
(fold basal)
3
2
1
0
*
#
#
#
*
%
%
%
LAB (μM) 0 2.5 5 10 0 2.5 5 10 0 2.5 5 10
PDGF - - - - + + + + - - - -
H2O2 - - - - - - - - + + + +

Figure 2. Effect of LAB on the PDGF-BB-induced intracellular ROS levels in rat VSMC. VSMCs were incubated in the absence or presence of varying doses of LAB for 1 h and stimulated with 10 ng/ml PDGF-BB for 10 min or 100 μM H_2O_2 for 1 min. Then the cells were incubated in the dark for 10 min with DCF-DA. Culture dishes were inspected with a fluorescence microscope imaging system, and the cellular fluorescence was visualized. A. Effect of LAB on intracellular ROS levels in VSMCs. A-1: Control, A-2: 2.5 μM LAB, A-3: 5 μM LAB, A-4: 10 μM LAB. B. Effect of LAB on intracellular ROS levels induced by 10 ng/ml PDGF-BB. B-1: PDGF-BB, B-2: PDGF-BB + 2.5 μM, B-3: PDGF-BB + 5 μM, B-4: PDGF-BB + 10 μM LAB. C. Effect of LAB on intracellular ROS levels induced by 100 μM H_2O_2. C-1: H_2O_2, C-2: H_2O_2 + 2.5 μM, C-3: H_2O_2 + 5 μM, C-4: H_2O_2 + 10 μM LAB. Values are means ± SD. *, $p< 0.001$ compared with the control; #, $p< 0.001$ compared with PDG-BB alone treated group; %, $p< 0.001$ compared with H_2O_2 alone treated group (n=5).


A.
pAkt
Akt
PDGF-BB (min) 0 5 10 20 30 60
pAkt/Akt
(fold basal)
6
1
PDGF-BB 0 5 10 20 30 60 (min)
10 ng/ml


B.
pAkt
Akt
LAB (μM) 0 0 1 5 10 20 30
PDGF-BB 10 ng/ml
pAkt/Akt
(fold basal)
3
1
LAB (μM) 0 0 1 5 10 20 30
PDGF-BB 10 ng/ml

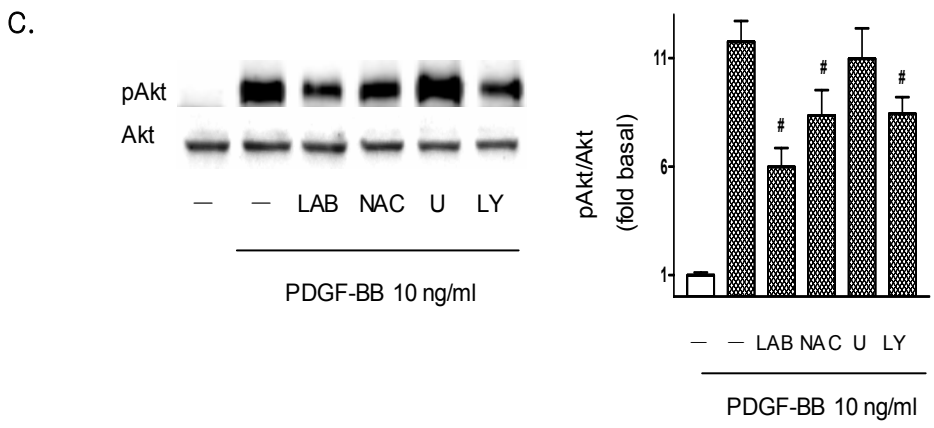
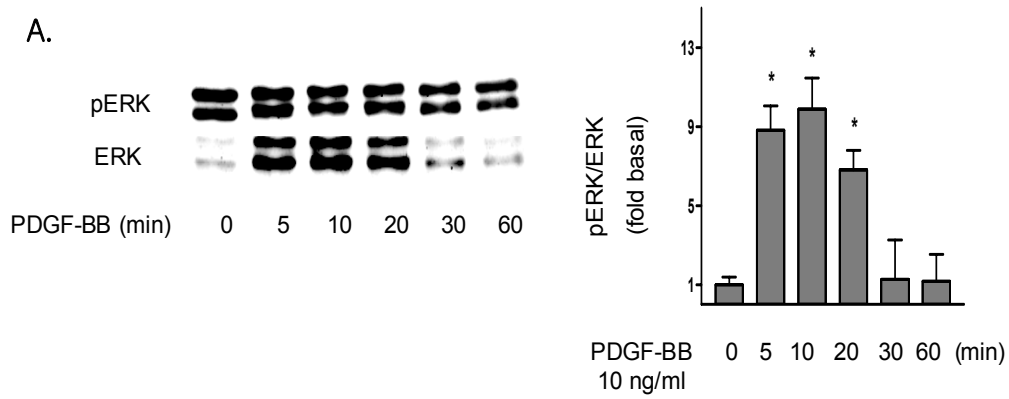
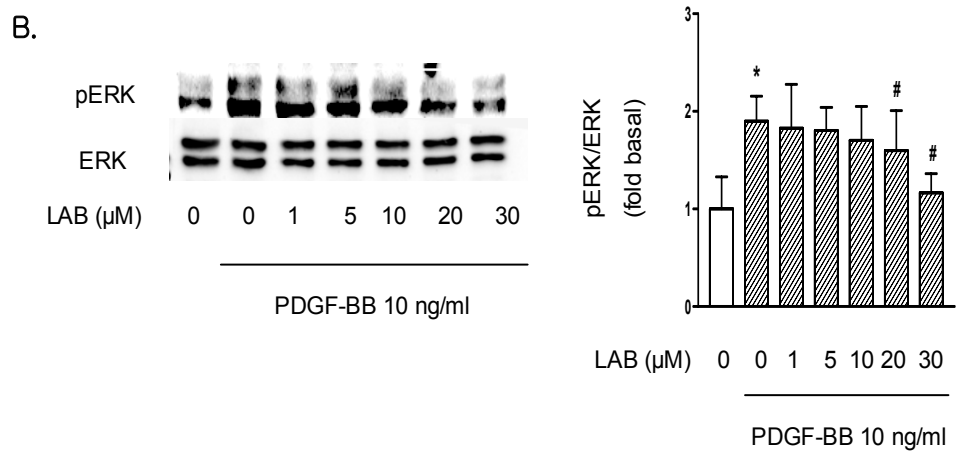

C.
pAkt
Akt
— — LAB NAC U LY
PDGF-BB 10 ng/ml
pAkt/Akt
(fold basal)
11
6
1
— — LAB NAC U LY
PDGF-BB 10 ng/ml

Figure 3. Effect of LAB on PDGF-BB-stimulated phosphorylation and the activity of Akt. A. VSMCs were treated with 10 ng/ml PDGF-BB for time periods ranging from 5 min to 1 h. B. Effect of LAB on PDGF-BB-induced Akt activation in VSMCs. VSMCs were pretreated with the indicated concentrations of LAB (1, 5, 10, 20 and 30 μM) for 1 h, then stimulated with 10 ng/ml PDGF-BB for an additional 10 min. C. Effect of various inhibitors on PDGF-BB-induced Akt activation in VSMCs. VSMCs were pretreated with 10 μM U0126, 10 μM SB203580, 40 μM LY294002 and 10 μM NAC and 30 μM LAB for 1h, and then stimulated with 10 ng/ml PDGF-BB for 10 min. Values are means ± SD. *, $p< 0.001$ compared with the control; #, $p< 0.001$ compared with the PDGF-BB alone treated group (n=3).


A.
pERK
ERK
PDGF-BB (min) 0 5 10 20 30 60
pERK/ERK (fold basal)
13
9
5
1
*
*
*
PDGF-BB 0 5 10 20 30 60 (min)
10 ng/ml


B.
pERK
ERK
LAB (μM) 0 0 1 5 10 20 30
PDGF-BB 10 ng/ml
pERK/ERK (fold basal)
3
2
1
0
*
#
#
LAB (μM) 0 0 1 5 10 20 30
PDGF-BB 10 ng/ml

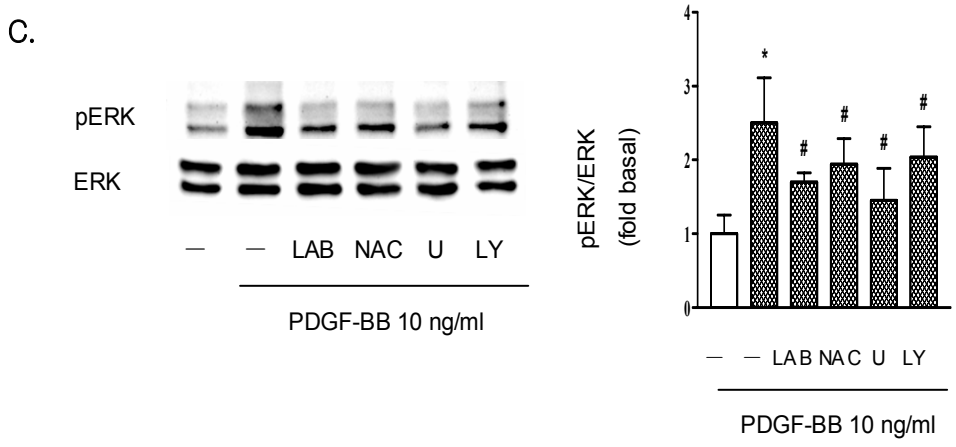

C.
pERK
ERK
— — LAB NAC U LY
PDGF-BB 10 ng/ml
pERK/ERK (fold basal)
4
3
2
1
0
*
#
#
#
— — LAB NAC U LY
PDGF-BB 10 ng/ml

Figure 4. LAB inhibits PDGF-BB-stimulated phosphorylation and activity of ERK 1/2. A. PDGF-BB increased ERK 1/2 posphorylation in VSMCs. VSMCs were treated with 10 ng/ml PDGF-BB for time periods ranging from 5 min to 1 h. B. Effect of LAB on PDGF-BB-induced ERK 1/2 activation in VSMCs. VSMCs were pretreated with the indicated concentrations of LAB (1, 5, 10, 20 and 30 μM) for 1 h, then stimulated with 10ng PDGF-BB for an additional 10 min. C. Effect of various inhibitors on PDGF-BB-induced ERK 1/2 activation in VSMCs. VSMCs were pretreated with 10 μM U0126, 10 μM SB203580, 40 μM LY294002 and 10 μM NAC and 30 μM LAB for 1h, and then stimulated with 10 ng/ml PDGF-BB for 10 min. Values are means ± SD. *, $p< 0.001$ compared with the control ; #, $p< 0.001$ compared with the PDGF-BB alone treated group (n=3).

IV. Discussion

Over the years many attempts have been made to develop treatments that inhibit the progress of cardiovascular diseases, such as atheroscleosis. VSMC hypertrophy is a critical determinant of atherosclerosis. The hypertrophic effect of PDGF-BB has been well recognized in various cell type, especially in VSMC. Moreover, PDGF-BB induces ROS. ROS have been implicated in the pathogenesis of a variety of human disease, including atherosclerosis. Therefore, inhibition of PDGF-BB-induced VSMC proliferation may contribute to prevent the development of cardiovascular diseases.

Previous nephrological studies have suggested that LAB prevent the incidence of various renal diseases [20, 28, 29, 30] which as a potent ROS scavenger. We assumed that LAB exerts a possible inhibitory effect against VSMC growth as ROS scavenger. Although the chemical structure of LAB is known (Table. 1), the effect and underlying mechanisms of LAB on cardiovascular disease remain largely unknown.

In this study, we demonstrated that LAB is a potent inhibiting agent of PDGF-BB-induced proliferation in rat VSMCs as a selectively inhibitor of signaling transduction cascade of the PDGF-BB and a potent ROS scavenger.

As shown in Figure. 1A, pretreatment with LAB caused remarkable

inhibition of PDGF-BB stimulated rat VSMC in a dose-dependent manner. . Also, VSMC proliferation by PDGF-BB is inhibited by radical scavenger, NAC. It suggests that PDGF-BB-induced VSMC proliferation has relation to intracellular ROS generation. Recent evidence suggests that ROS may function as second messengers for cytokine, involved interlukin-1 and tumor necrosis factor-α, and some growth factors that mediate the intracellular signal transduction pathways [10]. In particular, ROS have been shown to stimulate proliferation of VSMC and PDGF-BB requires the generation of hydrogen peroxide (H_2O_2). This study demonstrated that LAB showed an inhibitory effect in a concentration dependent manner for both PDGF-BB and H_2O_2 (Fig. 2). From these results, LAB has a free radical scavenging activity and it mediates its antiproliferative action. These LAB effects are similar with the results in mesangial cells of Lee et al [20].

In addition to Figure 1A, PDGF-BB-induced proliferation was inhibited by 20μM U0126 and 40μM LY294002 (Fig. 1B), but the JNK inhibitor I and SB202190, a p38 MAPK inhibitor had no effect (data not shown). These results suggest that PDGF-BB-induced intracellular signaling transduction pathway includes ERK 1/2 and PI3K pathway, and LAB has inhibitory effect of PDGF-BB-induced proliferation in VSMCs.

We attempt to identify the real molecular targets of LAB's effects in rat VSMCs using antiphospho-antibidies of two major intracellular signal pathways.

PDGF ß-receptors, that contain intrinsic tyrosin kinase activity, are dimerized and autophosphorylated on the tyrosin residues by their ligand. The phosphotyrosines on the receptor operate as high affinity binding sites for several molecules involved in the downstream propagation of signals [31]. This autophosphorylation is followed by the sequential activaton of Ras, Raf, MEK, ERK. ERK translocation to the nucleus initiates a proliferative response. Another molecule that is recruited by PDGF ß-receptors is phosphatudylinostol 3-kinase (PI3K). One of the downstream effectors of PI3K activation is Akt. It is known that PI3K is necessary for the mitogenesis and chmotaxis induced by PDGF-BB in VSMCs. It is known that PI3-kinase plays a pivotal role in mediating motility responses, including PDGF-induced chemotaxis and migration [32].

We demonstrated that the mount of whole ERK and Akt protein expression in rat VSMC was not altered in the absence or presence of LAB. But amounts of the phosphorylated ERK and Akt in rat VSMC were significantly inhibited by LAB in a dose-dependant manner (Fig.3B, 4B). The levels of phosphorylation of both ERK 1/2 and Akt were significantly inhibited by as little as 30 μM LAB. These results indicate that one of the main molecular targets of LAB may be located at a common point upstream of both ERK 1/2 and Akt.

PI3K signaling has been linked to cellular proliferation in various cell types [33,34], and this has been correlated with the activation of ERK 1/2 [35, 36]. A large

number of studies have shown PI3K to be either upstream [36] or downstream [37] of ERK 1/2 and to be activated as part of a parallel or independent pathway [38, 39]. In this study, PDGF-BB stimulation resulted in the 10-fold phosphorylation of P-Akt/Akt and the 1.5-fold activation/ phosphorylation of ERK1/2, which were inhibited by LY294002 (Fig. 4C). ERK 1/2 activation was inhibited by U0126 in the PDGF-BB-induced signaling pathway, but effect of U0126 lacks effect on P-Akt/Akt phosphorylation (Fig. 3C). It is suggests that activation of ERK 1/2 may occur downstream of Akt in this PI3K dependent signal pathway. Similar observations have been reported in both Chinese hamster ovary (CHO) and Cos7 cells [40, 41] and human vascular smooth muscle cells [42].

Our results suggest that LAB, a main active component of Danshen, has antiproliferative effect in rat VSMCs by blockade of PI3K, ERK 1/2 pathways in a redox sensitive manner.

V. Conclusion

This present study showed that the effect of LAB as an antioxidant on PDGF-BB-stimulation in rat VSMCs and the LAB effect is mediated by the ERK 1/2 and AKT-mediated signaling pathway.

1. PDGF-BB induced VSMC proliferation was inhibited by LAB in a concentration dependent manner.

2. LAB showed an inhibitory effect of ROS induced by PDGF-BB in VSMCs.

3. ERK 1/2 inhibitor U0126 and PI3K inhibitor LY294002 prevented PDGF-BB induced VSMCs proliferation.

Taken together, these data suggest that LAB has an antiproliferative effect. This effect is related to a ROS inhibition effect and also, ERK 1/2 and Akt pathways.

In conclusion, LAB, an active component of S.miltiorrhizae radix, blockade may be bebeficial to prevent VSMC proliferative disorders associated with atherosclerosis and restenosis after angioplasty.

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국문요약

PDGF-BB에 의해 유도되는 이상 증식된 혈관 평활근 세포에서의 항산화제로써의 Lithosperte-B의 역할

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단삼은 전통적인 민간요법으로 혈관성질환에 사용되어온 약물이다. LAB는 단삼의 주요 구성 성분으로써 본 연구에서는 LAB의 동맥 경화에서의 효과를 밝히고자 하였다. 혈관 평활근 세포의 이상 증식은 동맥 경화의 주요 원인이 되며, PDGF-BB의 자극은 이러한 이상 증식을 유발한다. 또한 PDGF-BB는 활성 산소종의 발생을 유도하는데, 많은 세포 내 신호전달 과정에 관여된다고 보고되고 있다.

LAB는 단삼의 주요 구성 요소로 활성산소 생성 억제제로 알려져 있다. 이 연구에서는 LAB가 PDGF-BB에 의한 세포의 증식과 활성

산소종의 발생을 억제하는 효과를 가지는지 알아보았다. 1) PDGF-BB에 의해 유도된 세포증식은 LAB 의 농도에 비례하여 억제된다. 2) PDGF-BB에 의해 활성 산소종의 생성이 유도되지만, LAB 와 NAC를 전처리해준 세포에서는 활성 산소종의 생성이 억제되었다. 3) Akt는 PDGF-BB에 의해 phosphorylation/ activation 되며, 4) ERK 1/2 또한 PDGF-BB 에 의해 phosphorylation/ activation 되는데, 이것은 NAC 또는 LAB 전처리에 의해 억제되어 나타난다.

이상의 결과로, LAB는 PDGF-BB에 의해 생성되는 활성 산소종과 그에 따른 혈관평활근 세포의 증식을 Akt와 ERK 1/2의 경로를 통하여 억제시키는 효과를 가짐을 알 수 있다. 따라서 LAB는 새로운 동맥경화의 치료제로써 사용될 수 있을 것이라고 생각된다.

핵심되는 말 : 동맥경화증, Lithospermate-B, PDGF-BB, 활성산소종, 항산화제.