# The Effect of Magnesium Lithospermate B on Endothelial Dysfunction in Diabetes Mellitus

So Hun Kim

Department of Medicine
The Graduate School, Yonsei University

# The Effect of Magnesium Lithospermate B on Endothelial Dysfunction in Diabetes Mellitus

Directed by Professor Hyun Chul Lee

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of Doctor of Philosophy

So Hun Kim

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This certifies that the Doctoral Dissertation of So Hun Kim is approved.

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Thesis Supervisor

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Thesis Committee Member #1

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Thesis Committee Member #2

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Thesis Committee Member #3

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Thesis Committee Member #4

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**ABSTRACT**

**The Effect of Magnesium Lithospermate B on endothelial dysfunction in Diabetes Mellitus**

So Hun Kim

*Department of Medicine*

*The Graduate School, Yonsei University*

(Directed by Professor Hyun Chul Lee)

Impaired function of the endothelium is thought to be a precursor of atherosclerosis and a predictor of future vascular events. In diabetes, endothelial vasodilator function is compromised and this characterizes a proatherogenic state. This study was designed to investigate the effects of magnesium lithospermate B (LAB) on endothelial dysfunction associated with diabetes mellitus in cultured endothelial cells and in an animal model of type 2 diabetes.

LAB effect on hyperglycemia induced changes in endothelial nitric oxide synthase (eNOS) activity, phosphorylation of eNOS at serine 1177 and apoptosis was examined in cultured human endothelial cells. In human aortic endothelial cells (HAECs), hyperglycemia inhitibed eNOS activity by 86 %. LAB (50 μM) treatment was able to significantly prevent the decrease in eNOS activity by hyperglycemia. eNOS phosphorylation was decreased by

hyperglycemia and this decrease was prevented by LAB treatment. High glucose treatment for 48 hours increased apoptosis by 2 folds in HAECs. LAB treatment was able to decrease apoptosis in high glucose treated HAECs at doses of 12.5 μM and greater. 25 μM treatment decreased apoptosis induced by hyperglycemia by 36%. LAB effect on hyperglycemia and cytokine induced THP-1 monocyte adhesion to endothelial cells was also examined. THP-1 monocyte adhesion to HUVECs were increased by hyperglycemia and TNF-α treatment. This was decreased by LAB treatment. As an animal model of type 2 diabetes, Otsuka Long-Evans Tokushima Fatty (OLETF) rats were treated with LAB (20mg/kg/day) for 20 weeks, starting at 12 weeks of age and compared with age-matched placebo treated OLETF rats. In OLETF rats, endothelium-dependent vasodilation was decreased compared to control lean Long-Evans Tokushima Otsuka (LETO) rats at 32 weeks of age. LAB treated OLETF rats showed improved endothelium-dependent vasodilation compared to OLETF rats treated placebo.

In conclusion, LAB showed favorable effects on hyperglycemia induced endothelial dysfunction in cultured endothelial cells and showed to have preventive effects on endothelial dysfunction in OLETF rats.

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**Key words** : magnesium lithospermate B, endothelial dysfunction, diabetes mellitus

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## I. INTRODUCTION

The endothelium is widely accepted to play important roles in maintaining a normal vascular tone and blood fluidity, reducing platelet activity and leukocyte adhesion, and limiting vascular inflammatory reactions[1]. Many evidences suggest that endothelial dysfunction predispose the development of atherosclerosis and is a predictor of future vascular events[2,3]. In diabetes, endothelial vasodilator function is compromised because of changed production of vasodilator and vasoconstrictor substances, particularly nitric oxide (NO)[4]. Dysfunction of endothelium-dependent vasodilation in diabetes characterizes a proatherosclerotic state[5]. The metabolic abnormalities that characterize diabetes, particulary hyperglycemia, free fatty acids, and insulin resistance, provoke molecular mechanisms that alter the function and structure of blood vessels.

These include increased oxidative stress, disturbances of intracellular signal transduction and activation of receptor for advanced glycation endproducts (RAGE). Consequently, there is decreased availability of NO, increased production of endothelin, activation of transcription factors of NF-kB and AP-1, and increased production of prothrombotic factors[6].

Endothelial nitric oxide (eNOS) dysregulation and free radical generations have been reported to be involved in the induction of diabetic endothelial dysfunction[7-9]. Impairment of endothelium-dependent vasorelaxation can be produced by elevation in blood glucose levels, as demonstrated in both animals and humans[10, 11]. In endothelial cells, high glucose induces production of reactive oxygen species (ROS) which can cause cellular dysfunction and even death[12, 13]. Endothelial apoptosis may contribute to the pathogenesis of atherosclerosis by initiating the disturbance of the integrity of the endothelium monolayer and it may also contribute to plaque erosion and enhance thrombus formation, the major players for the acute coronary syndromes[14]. In animals, endothelial apoptosis correlated with an impairment of endothelial vasodilator function[15]. Several antioxidants have been reported to have beneficial effects on eNOS activation[16] and anti-apoptotic effects in endothelial cells exposed to hyperglycemia[17, 18].

Diabetes is also associated with endothelial dysfunction associated with vascular inflammation[19, 20]. During atherogenesis, endothelial cells are activated and the expression of adhesion molecules at the luminal surface of the

endothelium are increased. Increased adhesion of monocytes to the activated endothelium and their accumulation and transformation into macrophages generate foam cell-derived fatty streaks that develop into advanced atherosclerotic lesions[21-23]. Diabetes further exacerbates endothelial dysfunction and increases endothelial adhesion of leukocytes[19, 20, 24]. Pathological endothelial changes are mediated in part by inflammatory cytokines such as TNF-α, which cause activation and increase adhesion[25, 26].

*Salvia miltiorrhizae* radix is a Chinese herbal medicine that has been traditionally used either alone or mixed with other herbs, to treat blood circulating diseases, such as cardiovascular disease, cerebrovascular disease, and chronic renal failure. Among caffeic acid and its oligomer condensates, magnesium lithospermate B (LAB) is the most common component in *Salvia* species and is abundant in their aqueous extracts[27]. LAB is known to have potent antioxidative[28] and antifibrotic effects[29]. Previous studies reported that LAB or Salvia miltiorrhizae's aqueous extracts salvage myocardium under hypoxia/reperfusion[30], improves endothelial dysfunction[31], exert preventive effects on atherosclerosis[32-35] and renal failure[36-38]. The effect of LAB on endothelial dysfunction in diabetes is currently unknown.

Previous reports of the effect of LAB as a potent antioxidant and its preventive effects on the initiation and progression of atherosclerosis in other conditions suggests that LAB may have a favorable effect on endothelial dysfunction in diabetes. Therefore, this study was set to examine whether LAB inhibited

hyperglycemia induced apoptosis in human endothelial cells and attenuated hyperglycemia induced inhibition of eNOS activity. Whether hyperglycemia and TNF-α induced monocyte adhesion to endothelial cells were altered by LAB and the action of LAB on endothelial dysfunction in OLETF rats were also examined.

## II. MATERIALS AND METHODS

### 1. Cell culture and high-glucose experiments

Human umbilical vein endothelial cells (HUVEC) and human aortic endothelial cells (HAEC) were obtained from Cambrex (Walkersville, MD). HUVECs and HAECs were maintained in EBM-2 growth media supplemented with EGM-2 bullet kit (Cambrex, Walkersville, MD) in a humidified atmosphere (5% $CO_2$, at 37°C). After the cell layer became near confluent subcultures were performed using trypsin-EDTA. For all experiments cells from the $3^{rd}$ to $7^{th}$ passage were used. EBM-2 growth media supplemented with 2% FBS and antibiotics were used for incubation of cells under experimental conditions. In experiments, Human endothelial cells were treated with media containing 5.5mmol/L or 30mmol/L of glucose for 48 hours. Experiments were performed with or without addition of LAB (6.25 - 50μM).

### 2. eNOS activity assay

eNOS activity in HAECs was measured using the Parameter™ Total NO/Nitrate/Nitrite assay kit (R&D Systems, MN) through the Griess Reaction, according to the manufacturer's instructions. HAECs were grown to confluence in EBM-2 medium with EGM-2 bullet kit in 24 well plates. The medium was then changed to EBM-2 medium with 2% FBS and antibiotics with 5.5 or 30mmol/l glucose and incubated for 48 hours. Drug treatment was started 1

hour prior to the medium change. After 48 hours treatment the cell culture supernatant was removed . 1ml of HEPES buffer with 1mM arginine was added and incubated for 1hour. The supernatant was harvested after the 1 hour incubation and NO production was measured.

**3. Adhesion assay**

Adhesion of THP-1 cells to HUVEC monolayer was assayed. HUVECs were plated on 6-well plates at the density of 1 x $10^5$ cells per well and cultured to 90% confluence in EGM-2 Bullet kit media. The cells were treated in normal glucose (5.5mM) or high glucose (30mM) conditions or with TNF-α (2ng/ml) for 48 hours. Drug treatment was started 1hour prior to media change. THP-1 cells in the exponential growth phase were washed with serum-free RPMI 1640 medium and suspended in the same medium (1 x $10^5$ Cells). One milliliter of the THP-1 suspension was added into the wells containing HUVEC monolayers and incubated for 20 min at 37℃. The unbound cells were washed three times with serum free RPMI 1640 medium, and the total number of adherent cells was counted in four randomly selected optical fields per well.

**4. Detection of cell apoptosis**

Apoptosis was detected by using the method of "Cell Death Detection ELISA Kit" (Roche Applied Science, Indianapolis, IN). This assay is based on the quantitative sandwich-enzyme-immunoassay principle using mouse monoclonal

antibodies directed against DNA and histones, respectively. It allows specific determination of mono- and oligonucleosomes in the cell lysates. When HUVECs were near confluent the media was changed to EBM-2 growth media supplemented with 2% FBS and antibiotics. Cells were incubated in high glucose (30mM) or normal glucose (5.5mM) conditions for 48 hours with or without the indicated drugs. Cell culture media was changed daily. The adherent cells were lysed by adding cell lysis buffer to the culture plates and after centrifugation supernatants were used for analysis. Measurement was done at 405nm wavelength.

**5. Western blot analysis**

After the indicated treatments, cells were briefly washed with ice-cold phosphate-buffered saline (PBS). Cells were then scraped in a lysis buffer that contained 20mM Tris-HCl (pH 7.6), 0.15M NaCl, 5mM EDTA, 0.5% Nonidet P-40, 1ul/ml DTT, and complete protease inhibitor mixture (Roche Applied Science). Cell debris were pelleted by centrifugation of samples at 13,000 rpm for 30minutes. Supernatants were then boiled with Laemmli sample buffer for 5minutes and proteins were resolved by 10% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with specific antibodies. Antibody binding was detected by enhanced chemiluminescence. Immunopositive bands were quantified by scanning densitometry. For nuclear extract preparation, cells were washed three times with ice-cold PBS and

resuspended in 200 μL of buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 1.5mM $MgCl_2$, 0.5 mM DTT, 1 mM AEBSF, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). After 10 min, nuclei were pelleted and suspended in 50 μL of buffer B (20mM HEPES (pH 7.9), 0.42 M KCl, 1.5mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, 1 mM AEBSF, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). After 30-min agitation at 4°C, the lysates were centrifuged and the supernatants were diluted with buffer C (20mM HEPES (pH 7.9), 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, 1 mM AEBSF, 1 μg/ml leupeptin, and 1 μg/ml aprotinin).

**6. Animals and experimental protocol**

Male Otsuka Long-Evans Tokushima fatty (OLETF) rats and non-diabetic control Long-Evans Tokushima Otsuka (LETO) rats were obtained from the Otsuka Pharmaceutical Company (Tokushima, Japan). The rats were maintained in an animal room controlled at 23 ± 2 ℃ and 55 ± 5% room humidity, under a 12h-light 12h-dark cycle. All rats were maintained on standard rat chow (Samyang rat chow, Seoul, Korea) and tap water ad libitum. The animals were divided into 3 groups at 12 weeks of age: a control group (LETO), placebo treated-diabetes group (OLETF + Placebo), and LAB-treated diabetes group (OLETF + LAB). LAB was dissolved in water and was given daily to OLETF rats from 12 weeks of age at a daily dose of 20mg/kg via oral gavage. Placebo treated animals were given the same volume of distilled water daily via oral

gavage. An oral glucose tolerance tests was performed at the age of 29 weeks by oral administration of glucose solution (2 g / kg bodyweight ) after 16 hours of fasting. Blood samples were taken at 0, 30, 60, 90, and 120 minutes from the tail vein. Blood glucose concentrations were determined using a glucometer (Accu-Check, Roche diagnostics, Switzerland). Blood pressure was measured from the femoral artery using an isometric force displacement transducer. The animals were killed at the age of 32 weeks. Blood samples were obtained for blood chemistry. The thoracic aorta was excised and used for vascular function studies.

**7. Biochemical analysis**

Blood samples were immediately centrifuged at 5,000g for 5 minutes and serum was separated and stored in a -70℃ freezer. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) was measured by an IFCC UV method. Serum total cholesterol, triglyceride, free fatty acid level was measured by an enzymatic method and High density lipoprotein (HDL)-cholesterol was measured using a selective inhibition method.

**8. Vascular function study**

The thoracic aorta (0.6 to 0.8 cm outside diameter) was isolated and cut into strips 2mm long with special care taken to preserve the endothelium and bathed in 15 ml Krebs-Henseleit Solution (glucose 11.1mM, $NaHCO_3$ 25mM,

NaCl 119mM, KCl 4.6mM, $MgSO_4$ 1.2mM, $KH_2PO_4$ 1.2mM, $CaCl_2$ 2.5mM, EDTA 0.01mM [pH 7.4]) equilibrated with 95% O2 and 5% CO2 and maintained at 37℃. The rings were suspended under 1g of tension and preconstricted by adding $10^{-7}$M L-phenylephrine. After a plateau was attained, the strips were exposed to acetylcholine ($10^{-9}$~ $10^{-5}$ M) to evaluate endothelium-dependent vasodilation and sodium nitroprusside ($10^{-11}$ ~ $10^{-7}$ M) to measure endothelium-independent vasodilation and dose-response curves were constructed accordingly.

**9. Statistical methods**

Results are expressed as means ± SD and data in figures are expressed as means ± SE. Data were analyzed by analysis of Variance plus Bonferonni multiple comparison tests. Values of $P<0.05$ were considered significant.

## III. RESULTS

### 1. Effect of LAB on eNOS activity

HAECs were incubated in EBM-2 media with 5.5mM or 30mM glucose for 48 hours in the presence or absence of LAB 50uM. NOS activity was significantly reduced (by 83%) in HAECs incuated in high glucose containing medium. The decrease in NOS acitivity after high glucose treatment was significantly inhibited by LAB treatment (Fig 1). As eNOS activity can be increased by phosphorylation of eNOS at Serine 1177, we examined phosphorylation of eNOS in LAB treated cells cultured in a hyperglycemic condition. The phosphorylation of eNOS was significantly decreased after 48hours of high glucose treatment as shown in Figure 2. LAB treatment inhibited the decrease in phosphorylation of eNOS at the serine 1177 site induced by hyperglycemia.

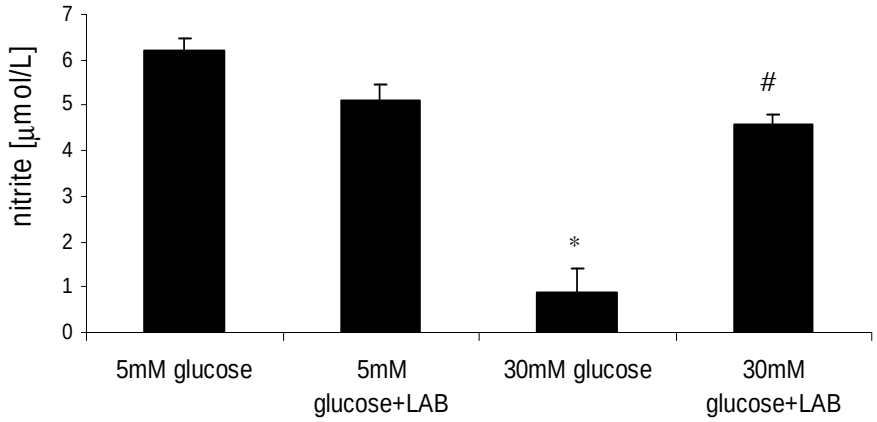



**Figure 1. Effects of LAB on NOS activity in HAECs.** Cells were incubated in normoglycemic (5.5mM glucose) or hyperglycemic (30mM glucose) media alone or with LAB (50uM) for 48 hours. LAB was added 1hour prior to exposure to normoglycemic or hyperglycemic medium. NOS activity was examined by measuring the production of nitrite as described in Methods. *: $p<0.05$ compared with cells incubated in 5.5mM glucose, $^{\#}$: $p<0.05$ compared with cells incubated in 30mM glucose. n=5 for each group.

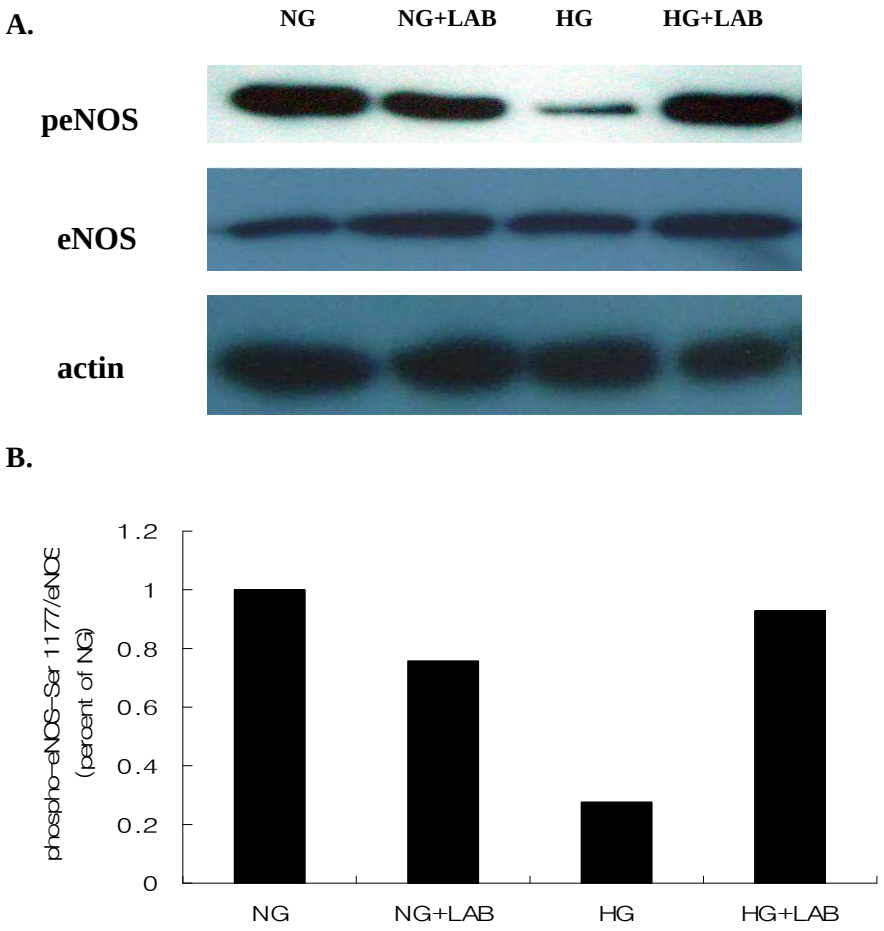
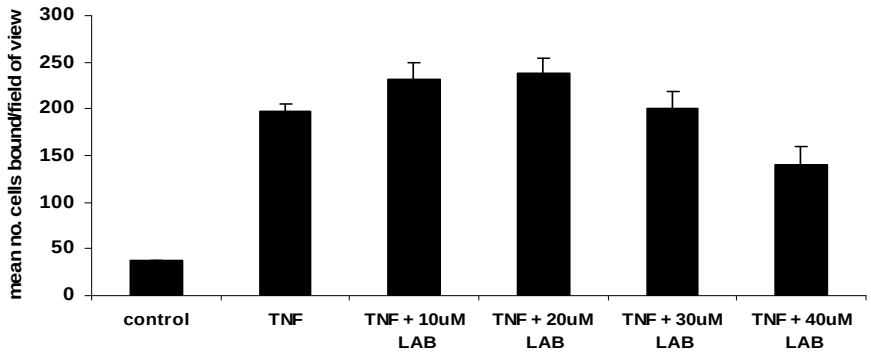



**Figure 2. Effect of LAB on eNOS phoshphorylation in human endothelial cells.** HAECs were treated with normal glucose (NG: 5.5mM) or high glucose (HG: 30mM) for 48 hours with or without LAB (50uM). Western blot analysis with serine 1177 phsopho-eNOS, eNOS, and actin antibodies were performed as described in Methods. Representative western blots (A) and relative phosphorylation of eNOS at Serine 1177 normalized to total eNOS in comparison to normal glucose condition are shown (B).

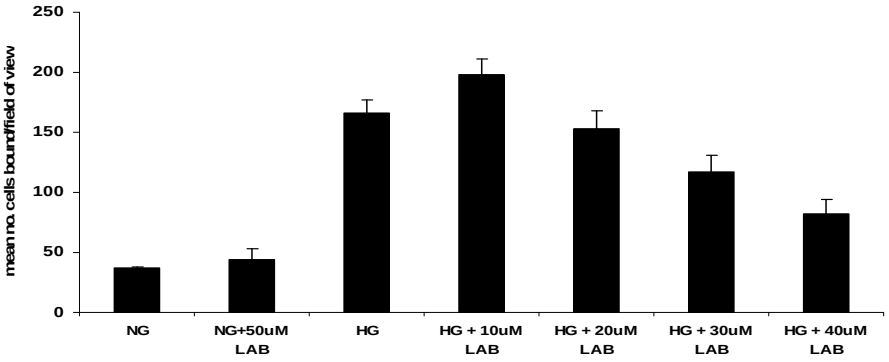
## 2. Effect of LAB on monocyte adhesion to endothelial cells

Treatment with high glucose for 48 hours significantly increased THP-1 cell monocyte adhesion to HUVECs by 4 times. LAB dose dependently decreased the monocyte adhesion to HUVECs induced by hyperglycemia. LAB also decreased the monocyte adhesion to HVUECs induced by TNF-α (Fig 3). To examine whether the decreased monocyte adhesion was associated with attenuated adhesion molecule expression in HUVECs, the effect of LAB on TNF-α induced ICAM-1 and VCAM-1 expression was examined. LAB decreased TNF--α induced VCAM-1 expression in HUVECs (Fig 4). NF-kB is a transcription factor that regulates expression of VCAM-1, which LAB was able to alter. Therefore, to determine whether LAB affects NF-kB activation, nuclear translocation was examined by a western blot analysis from the nuclear extract. LAB inhibited TNF-α induced NF-kB nuclear translocation (Fig 5).

**A**


mean no. cells bound/field of view
300
250
200
150
100
50
0
control
TNF
TNF + 10uM LAB
TNF + 20uM LAB
TNF + 30uM LAB
TNF + 40uM LAB

**B.**


mean no. cells bound/field of view
250
200
150
100
50
0
NG
NG+50uM LAB
HG
HG + 10uM LAB
HG + 20uM LAB
HG + 30uM LAB
HG + 40uM LAB

**C**

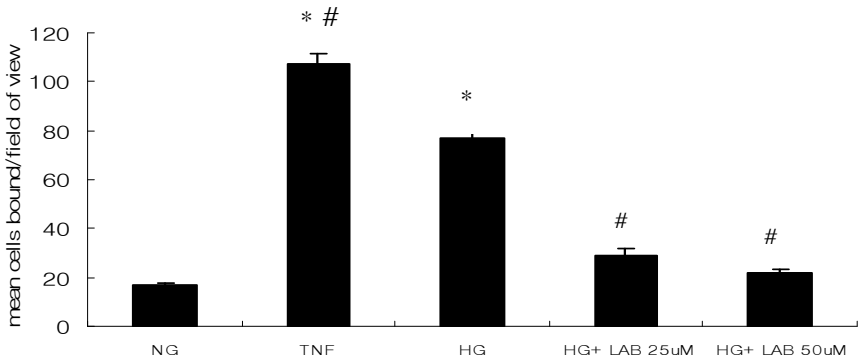
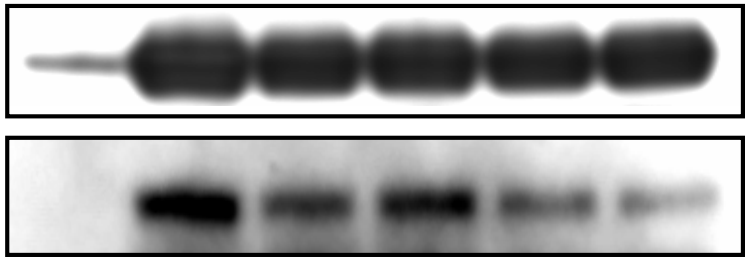
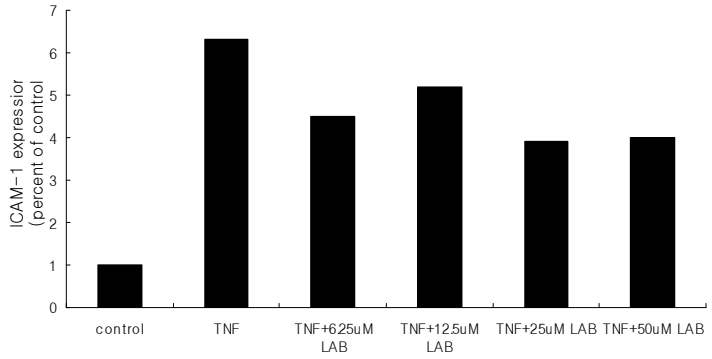

mean cells bound/field of view
120
100
80
60
40
20
0
* #
*
#
#
NG
TNF
HG
HG+ LAB 25uM
HG+ LAB 50uM

**Figure 3. Effect of LAB on THP-1 cell adhesion.** An in vitro adhesion assay was performed to measure firm THP-1 monocyte adhesion on TNF-α stimulated (TNF) (A) or high glucose (HG, 48hours) treated (B,C) HUVEC with or without LAB treatment. The total numbers of attached cells counted on 4 randomly selected microscopic fields are means ± SE. $^{*}$: $p<0.05$ compared with cells incubated in 5.5mM (NG), $^{\#}$: $p<0.05$ compared with cells incubated in 30mM (HG). n=5 for each group.

**A**


ICAM-1
VCAM-1
Control
0
6.25
12.5
25
50
TNF-α (2ng/ml) + LAB (μM)

**B**


ICAM-1 expressior
(percent of control
0
1
2
3
4
5
6
7
control
TNF
TNF+6.25uM LAB
TNF+12.5uM LAB
TNF+25uM LAB
TNF+50uM LAB

**C**

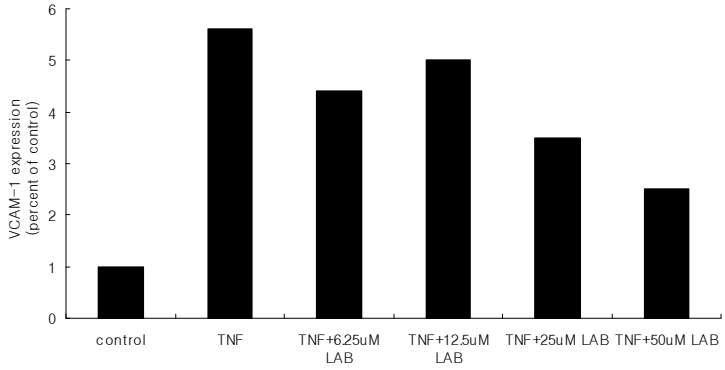
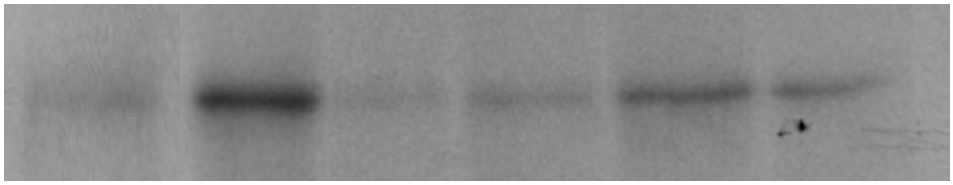

VCAM-1 expression
(percent of control)
0
1
2
3
4
5
6
control
TNF
TNF+6.25uM LAB
TNF+12.5uM LAB
TNF+25uM LAB
TNF+50uM LAB

**Figure 4. Effect of LAB on TNF-α induced ICAM-1 and VCAM-1 expression in HUVECs.** Western blot analysis was performed after 48 hours treatment with TNF-α (2ng/ml) with different concentrations of LAB (6.25 μM, 12.5 μM, 25 μM, 50 μM). LAB was added 1hour prior to TNF-α treatment. Representative western blot (A), ICAM-1 expression (B) and VCAM-1 expression is shown. TNF: tumor necrosis factor-α.



| Control | 0 | 6.25 | 12.5 | 25 | 50 |
|---|---|---|---|---|---|

**TNF-α (5ng/ml) + LAB (uM)**

**Figure 5. Effect of LAB on TNF-α induced nuclear p65 expression in HUVECs.** Western blot analysis of the nuclear extracts were performed after 3 hours treatment with TNF-α (5ng/ml) with different concentrations of LAB. LAB was added 1hour prior to TNF-α treatment.

### 3. Effect of LAB on hyperglycemia induced endothelial apoptosis

HUVECs were cultured in normoglycemic or hyperglycemic media for 48hours with or without LAB (12.5, 25, and 50 μM) or alpha lipoic acid (50 and 100μM). Drug treatment was initiated 1 hour before treatment of the hyperglycemic media. HUVEC apoptosis was increased significantly by two folds in hyperglycemic conditions and this was significantly prevented by LAB treatment (Fig 6). The significant inhibition of hyperglycemia induced apoptosis was seen at LAB concentrations of 12.5 μM and higher, with 36% maximum prevention at concentration of 25μM. Alpha lipoic acid was also associated with similar effects in preventing hyperglycemia induced apoptosis. LAB and alpha lipoic acid both showed no significant effect on apoptosis in normoglycemic conditions.

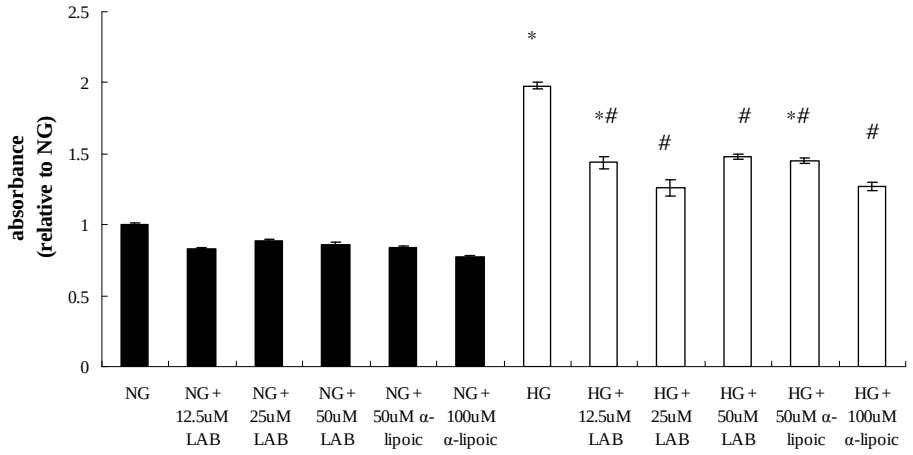



**Figure 6. Effects of LAB on high-glucose induced apoptosis in cultured HUVECs.** HAECs were treated with normal glucose (NG: 5.5mM) or high glucose (HG: 30mM) for 48 hours with or without LAB (12.5, 25, and 50 μM) and alpha lipoic acid (α-lipoic; 50uM and 100uM). Apoptosis was analyzed by measuring the level of cytosolic histone-bound DNA fragments using a cell death ELISA kit. The values are means ± SEM of 5 samples. * $p<0.05$ compared with cells incubated in 5.5mM (NG), $^{\#}$: $p<0.05$ compared with cells incubated in 30mM (HG).

**4. Effect of LAB treatment on vascular function in OLETF rats.**

OLETF rats were treated with LAB or placebo starting at 12 weeks, for 20 weeks. LETO rats were used as control. At 32 weeks, the animals were sacrificed and the thoracic aorta was isolated for vascular function evaluation. The body weight of the OLETF rats were significantly higher compared to the LETO control rats. LAB treatment showed no effect on the body weight in OLETF rats (Fig 7). An oral glucose tolerance test at 29 weeks showed significant glucose intolerance in OLETF rats compared to LETO rats. LAB treatment did not have any positive effects on glucose intolerance compared with placebo (Fig 8). There was no difference in lipid profiles or mean blood pressure in LAB treated OLETF rats compared with placebo treated rats (Table 1). Endothelium-dependent vasodilation was assessed by incubating endothelium intact vascular rings to increasing doses of acetylcholine ($10^{-9}$ M to $10^{-5}$ M). Endothelium-dependent vasodilation was significantly reduced in placebo treated OLETF rats compared to LETO rats (76.4% maximal relaxation in LETO vs. 50.0% maximal relaxation in placebo treated OLETF rats, $p<0.05$). LAB treatment significantly improved endothelium-dependent vasodilatory function in OLETF rats. Vascular rings of OLETF rats treated with LAB that were exposed to higher doses ($10^{-6}$ M and $10^{-7}$ M) of acetylcholine showed significantly increased vessel relaxation compared to those of OLETF rats treated with placebo (Fig 9A). Endothelium-independent vasodilation was assessed by incubating the vascular rings in increasing doses of sodium nitroprusside ($10^{-11}$ M to $10^{-7}$ M). Endothelium-independent vasodilation was slightly reduced in OLETF rats but LAB treated groups showed no significant difference compared to those treated with placebo (Fig 9B).

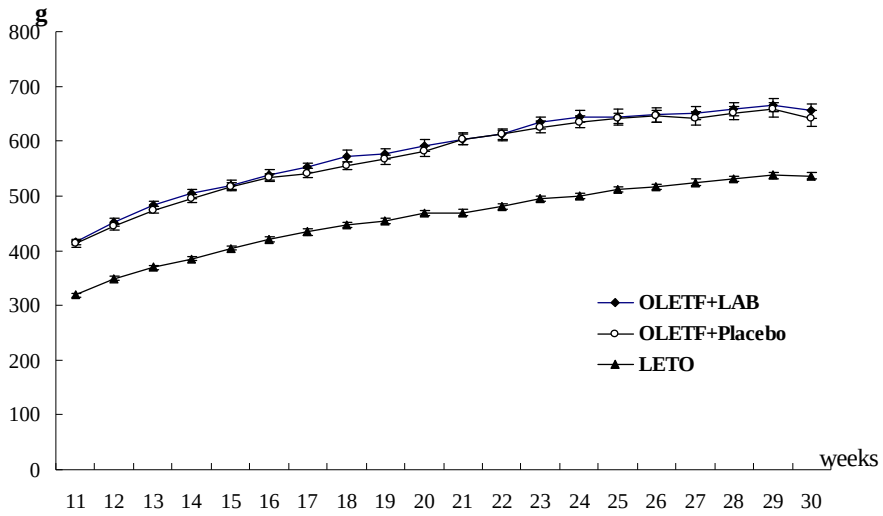



**Figure7. Change of body weight in the 3 groups of animals.** Body weight of the OLETF rats treated with LAB (OLETF + LAB: ●), OLETF rats treated with placebo (OLETF + Placebo: ○) and LETO rats (LETO: ▲) were measured weekly.

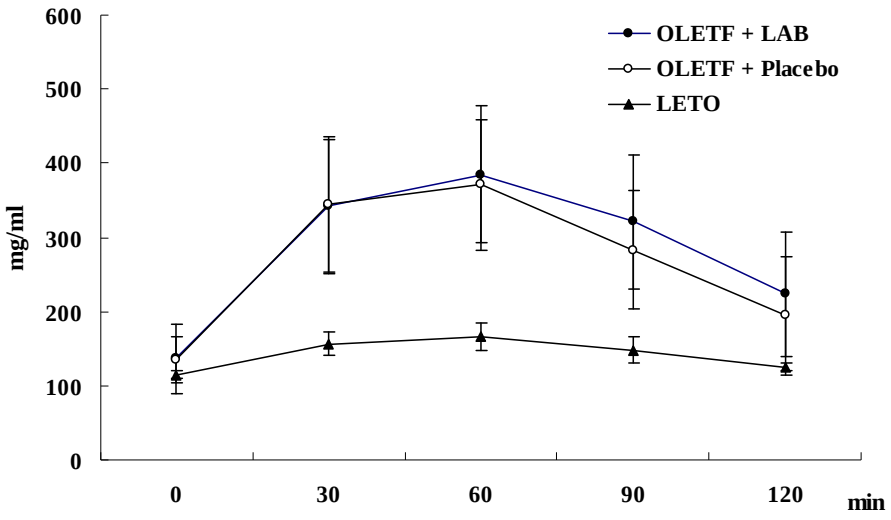


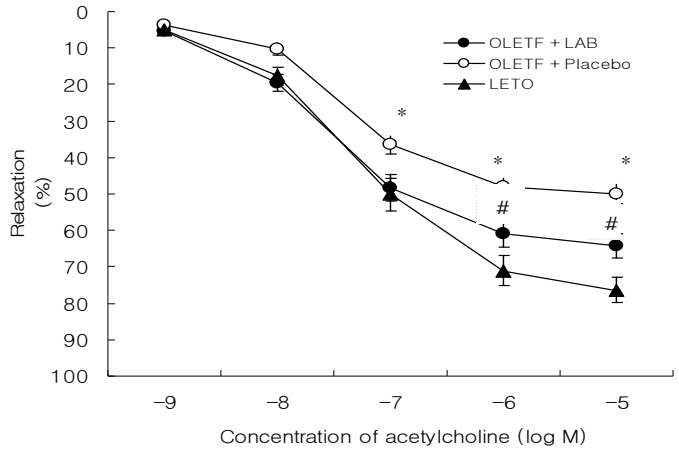

**Figure 8. Blood glucose concentrations during the oral glucose tolerance test.** Blood glucose concentration was measured during an oral glucose tolerance test at 29weeks in OLETF rats treated with LAB (OLETF + LAB: ●), OLETF rats treated with placebo (OLETF + Placebo:○) and LETO rats (LETO: ▲).

**Table 1. Clinical characteristics at 30 weeks of age in LETO rats, OLETF rats treated with LAB and OLETF rats treated with placebo.**

| | LETO | OLETF + LAB | OLETF + Placebo | P** |
|---|---|---|---|---|
| AST (IU/L) | 153.4±14.6 | 103.8±28.5* | 114.4±17.1* | 0.02 |
| ALT (IU/L) | 56.0±7.3 | 58.0±6.2 | 53.2±3.3 | 0.45 |
| T.chol (mg/dl) | 105.4±6.7 | 129.5±22.1 | 129.4±22.5 | 0.07 |
| TG (mg/dl) | 24±4.3 | 160.5±66.6* | 170.2±71.6* | 0.01 |
| HDL-chol (mg/dl) | 28.8±1.3 | 38.0±7.0* | 39.2±5.4* | 0.02 |
| FFA (μEq/L) | 456.6±87.1 | 764.8±255.7 | 908.2±256.5* | 0.03 |
| Blood pressure (mmHg) | 111.6±4.5 | 114.0±4.0 | 119.5±5.7 | 0.19 |

Data are mean±SD. T.chol; Total cholesterol, TG; Triglyceride, HDL-chol; High density lipoprotein- cholesterol, FFA; Free fatty acid. OLETF + LAB; OLETF rats treated with LAB, OLETF + Placebo; OLETF rats treated with placebo, *; $p<0.05$ vs LETO, **; p value by ANOVA

**A.**




**B.**

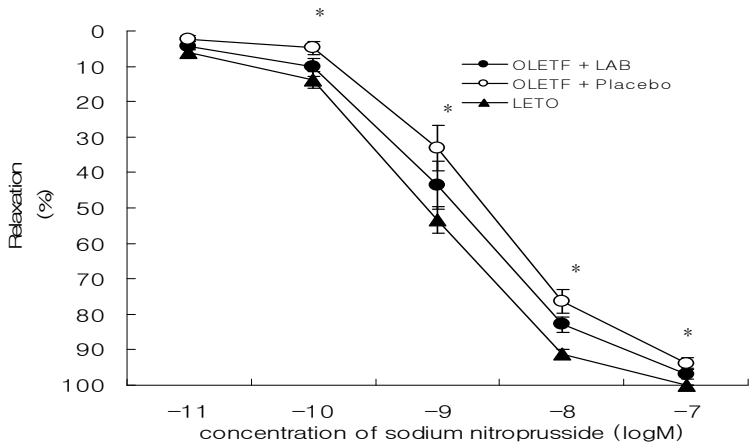



**Figure 9. Effects of LAB treatment on vasodilatory responses to acetylcholine (A) and sodium nitroprusside (B) after phenylephrine preconstruction of aortic segments.** Vasorelaxation was measured using an isometric force displacement transducer. Data are expressed as mean ± SEM (n=8 per group). *: P<0.05 vs LETO group; #: P<0.05 vs Placebo treated OLETF group.

## IV. DISCUSSION

The pathogenesis of atherosclerosis involves endothelial dysfunction, infiltration of monocytes, activation of monocytes into macrophages and smooth muscle cell proliferation[39]. Endothelial dysfunction is an early step in the pathogenesis of atherosclerosis and is a feature of diabetes[2, 3]. In diabetes, hyperglycemia and inflammatory stress is a contributing factor in the pathogenesis of atherosclerosis[6, 19]. The production of nitric oxide, which plays an important role in maintaining normal vascular function, seems to be decreased in diabetes[4]. In this study, we investigated the effects of LAB, an active component of *Salvia miltiorrhizae*, on endothelial dysfunction associated with diabetes mellitus in cultured endothelial cells and in an animal model of type 2 diabetes. LAB was able to improve eNOS activity which was decreased by hyperglycemia. LAB decreased hyperglycemia and TNF-α induced monocyte-endothelial cell adhesion and prevented hyperglycemia induced apoptosis in cultured human endothelial cells. In OLETF rats, treatment of LAB for 20 weeks starting at 12 weeks of age, significantly improved endothelial function compared to placebo treated OLETF rats.

*Salvia miltiorrhizae* radix is a Chinese herbal medicine widely used for the treatment of atherosclerosis-related disorders and diabetic complications. LAB is a phenolic compound abundant in the aqueous extract of *Salvia miltiorrhizae* and has been shown to have potent antioxidative [28, 40, 41] and antifibrotic effects[29]. The free radical scavenging effect was examined previously in our lab using the xanthine and xathine oxidase reaction system which chemically induces superoxide or $H_2O_2$. LAB was able to potently scavenge ROS dose dependently up to the concentration of 50 uM, which showed a 96% reduction in ROS. Since,

potent antioxidants scavenging free radicals can be clinically important for preventing and repairing damage to vascular endothelial cells and restoring vascular functions[2], it seemed possible that LAB may have a protective role on the endothelial dysfunction associated with diabetes. In previous studies, Kamata et al. found that LAB could endothelium-dependently relax the noradrenaline-precontracted aorta [42]. Wu et al. reported that the water-soluble antioxidant-rich fraction of Salvia miltiorrhiza was able to reduce atherosclerotic area in the abdominal aorta and reduce endothelial damage by 53% in cholesterol-fed rabbits. The decrease in atherosclerosis was thought to be associated with its antioxidant potential[35]. In this study, we show that LAB has preventive effects on multiple aspects of the dysfunctional endothelium seen in diabetes mellitus. LAB improved eNOS activity and prevented apoptosis in endothelial cells incubated in hyperglycemic media. LAB was able to attenuate the monocyte-endothelial adhesion activated by hyperglycemia and TNF-α in cultured endothelial cells, which is thought to be an early step in the development of atherosclerosis. In an animal model of type 2 diabetes, LAB had preventive effects on endothelial dysfunction.

In diabetes, endothelial vasodilator function is compromised because of changed production of vasodilator substances, particularly nitric oxide (NO)[4]. Endothelial production of NO plays an important role in preventing vascular disease through the regulation of thrombosis, inflammation, vascular tone and remodeling[22]. Endothelium-dependent vasodilation is impaired in both microcirculation and macrocirculation during acute hyperglycemia in both normal subjects[43, 44] and diabetic patients[45, 46], suggesting that NOS activity may be chronically impaired in diabetic patients. The effect of hyperglycemia is

mediated in large part by the state of enhanced oxidative stress, which is not counter-balanced by endogenous antioxidants[47]. Oxidative stress in the vasculature has been suggested to contribute to the development of endothelial dysfunction via different mechanisms including LDL oxidation, NO scavenging, or oxidation of tetrahydrobiopterin, a critical cofactor of eNOS. Antioxidants may interfere with these processes and protect NO formed in the endothelium. For instance, ascorbic acid improves tetrahydrobiopterin availability in the vasculature and α-tocopherol exerts a direct stimulatory effect on eNOS activation via serine 1177 phsophorylation[16].

In this study, LAB which is a potent antioxidant was also able to stimulate eNOS activation via serine 1177 phosphorylation in a hyperglycemic condition, where eNOS serine phosphorylation is inhibited. This leaded to increased eNOS activity by LAB in high glucose treated HAECs. This beneficial effect on eNOS activity was associated with prevention of endothelial dysfuncition in OLETF rats. The upstream event that leads to serine 1177 phosphorylation of eNOS by LAB needs to be evaluated in further studies. A possible mechanism involves the hexosamine pathway. Hyperglycemia induces overproduction of superoxide by the mitochondria and results in hexosamine pathway activation[48]. Du et al.[7] reported that hyperglycemia inhibited eNOS activity in cultured bovine aortic endothelial cells by activating the hexosamine pathway via mitochondrial overproduction of superoxide, which increases eNOS modification by GlcNAC and decreases eNOS serine phosphorylation in a reciprocal manner. The reciprocal modification was reported to occur specifically at Ser1177, the Akt phsophorylation site responsible for the activation of eNOS[49, 50]. Preliminary data show that hyperglycemia increases O-GlcNAC modification of eNOS in

HAECs and LAB treatment was able to inhibit O-glcNAC modification of eNOS. This finding supports the possible involvement of the alteration of hyperglycemia induced hexosamine pathway activation as the upstream mechanism although further investigation is needed.

In addition to the changes in endothelium-dependent vasodilation, ROS have also been studied in relation to leukocyte-endothelial interactions. Leukocyte adhesion and penetration of the endothelium can be initiated by a number of inflammatory cytokines, and is mediated by membrane adhesion molecules expressed on leukocytes and their respective vascular ligands. Among them, VCAM-1 and ICAM-1 are considered of particular importance. In general, normal vascular endothelial cells express very low levels of these molecules, which become much higher in the presence of hyperglycemia and vascular inflammation[2]. Increased adhesion of monocytes to the activated endothelium and their accumulation and transformation into macrophages generate foam cell-derived fatty streaks that develop into advanced atherosclerotic lesions[21-23]. Exogenous antioxidants have consistently shown in vitro and in vivo the capacity to reverse this pathological situation[51-54]. High glucose medium and TNF-α treatment to HUVECs increased leukocyte adhesion significantly. LAB was able to inhibit this process, which would lead to beneficial effects on prevention of atherosclerotic lesions. TNF-α was able to increase expression of VCAM-1 and ICAM-1 in HUVECs and LAB abrogated TNF-α induced expression of VCAM-1 but not ICAM-1 in HUVECs. ICAM-1 is involved in adhesion of various leukocytes, whereas VCAM-1 participates primarily in monocyte and lymphocyte adhesion and these cells are specifically found in atherosclerotic lesions[55]. It has been suggested that VCAM-1 gene transcription

and expression are regulated through an antioxidant-senstivie mechanism in HUVEC[56]. By actively inhibiting NF-kB mobilization, antioxidants like N-acetyl cysteine or pyrrolidine dithiocarbamate selectively reduced TNF-α induced VCAM-1 expression[57]. This seems to be the case for LAB also, since LAB was able to inhibit NF-kB mobilization and decrease VCAM-1 expression in HUVECs. Whether there is difference in the upstream mechanisms compared to other antioxidants should be further evaluated in future studies.

In endothelial cells, high glucose induces production of reactive oxygen species (ROS) which can cause cellular dysfunction and even death[12, 13]. Endothelial apoptosis may contribute to the pathogenesis of atherosclerosis by initiating the disturbance of the integrity of the endothelium monolayer and it may also contribute to plaque erosion and enhance thrombus formation, the major players for the acute coronary syndromes[14]. In animals, endothelial apoptosis correlated with an impairment of endothelial vasodilator function[15]. Several antioxidants have been reported to have anti-apoptotic effects in endothelial cells exposed to hyperglycemia[17, 18]. LAB, which has antioxidant potential, also was able to prevent apoptosis in endothelial cells exposed to hyperglycemia.

In OLETF rats, LAB treatment for 20weeks showed no favorable effects in glucose tolerance, body weight, lipid parameters and blood pressure compared with placebo treatment. Previous studies with LAB treatment at a dose of 20mg/kg/day in OLETF rats did not cause any cytotoxicity (unpublished data, Lee GT et al.). which was consistent with this study showing no cytotoxic effect.

The mean body weight of LAB treated OLETF rats were not different from placebo treated OLETF rats and LAB treatment did not cause any elevation in AST or ALT levels in OLETF rats. Although the testing of the effect of different doses of LAB (1-20mg/kg/day) showed similar effects at 10mg/kg/day and 20mg/kg/day (unpublished data, Lee GT et al.), since the bioavailability of LAB is relatively low[58], we used the maximally tolerated dose for maximal effect. The endothelium dependent vasodilation was significantly decreased in OLETF rats fed placebo compared to control lean LETO rats, and long term LAB treatment was able to attenuate the decrease and improve endothelium-dependent vasodilation in OLETF rats. Since reactive oxygen species is implicated in impaired endothelium-dependent relaxation in response to acetylcholine in aortic rings from diabetic animals[59], the antioxidant effect of LAB could play an important role in the improvement of acetylcholine induced relaxation of LAB treated OLETF rats seen in this study. The increased eNOS activity by LAB in endothelial cells exposed to hyperglycemia, which was demonstrated in HAECs should also play a role in the effects on endothelium-dependent vasodilation.

In conclusion, LAB showed favorable effects on hyperglycemia induced endothelial dysfunction in cultured endothelial cells and showed to have preventive effects on endothelial dysfunction in OLETF rats. LAB may be a promising drug for the prevention and treatment of endothelial dysfunction associated with diabetes mellitus.

## V. CONCLUSION

LAB was able to improve eNOS activity which was decreased by hyperglycemia by increasing phosphorylation of eNOS at Ser 1177. LAB decreased hyperglycemia and TNF-α induced monocyte-endothelial cell adhesion and this was associated with a decrease in NF-kB activation and VCAM-1 expression in endothelial cells. LAB was able to prevent hyperglycemia induced apoptosis in part by its antioxidant potential. In OLETF rats, treatment of LAB for 20 weeks starting at 12 weeks of age, significantly improved endothelial function compared to placebo treated rats.

In summary, LAB showed favorable effects on hyperglycemia induced endothelial dysfunction in cultured endothelial cells and showed to have preventive effects on endothelial dysfunction in OLETF rats. LAB may be a promising drug for the prevention and treatment of endothelial dysfunction associated with diabetes mellitus.

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**ABSTRACT(IN KOREAN)**

# Magnesium Lithospmerate B 가 당뇨병성 혈관내피세포 기능장애에 미치는 영향

<지도교수 이현철 >

연세대학교 대학원 의학과

김소헌

혈관내피세포 기능장애는 동맥경화의 전구단계이자 미래의 대혈관 합병증의 예측과 관련이 있다. 당뇨병에서는 혈관내피세포 기능장애가 나타나며 이는 동맥경화를 조장하는 특성을 가지게 된다. 이 연구에서는 Magnesium lithospermate B (LAB)가 당뇨병과 관련된 혈관내피세포 기능장애에 미치는 영향을 혈관내피세포와 제 2형 당뇨병 모델 동물인 Otsuka Long-Evans Tokushima Fatty (OLETF) 쥐를 이용하여 규명하고자 하였다.

고혈당에 의한 endothelial NOS (eNOS)의 활성 및 eNOS 의 serine 1177 위치의 인산화의 감소 및 세포 사멸에 대한 LAB의 효과를 살펴보았다. 고혈당 및 TNF-α에 의한 THP-1 단핵구와 혈관 내피세포의

부착에 대한 LAB의 효과도 관찰하였다. 제 2형 당뇨병 동물모델에서의 LAB 효과를 보기 위하여 OLETF 쥐 모델을 사용하여 12 주부터 LAB (20mg/kg/day) 또는 위약을 20주간 투여하였고 정상 대조군인 Long-Evans Tokushima Otsuka (LETO) 쥐를 비교하였다. 32주에 동물들을 희생하고 흉부대동맥을 분리하여 혈관 확장능을 확인하였다.

인간대동맥 내피세포에서 고혈당은 eNOS의 활성을 86% 감소시켰다. 50μM의 LAB는 고혈당에 의한 eNOS 활성의 감소를 유의하게 억제하였다. LAB에 의해 고혈당에 의한 eNOS 의 serine 1177 인산화의 감소가 회복되었다. 48시간 동안 HAEC을 48시간 동안 고혈당에 노출시켰을 때 세포 사멸은 2배 증가 하였다. LAB는 고혈당에 의한 세포사멸을 12.5uM 이상의 농도에서 예방하였다. LAB는 고혈당과 TNF-α에 의한 THP-1 단핵구와 혈관내피세포 부착의 증가를 감소시킬 수 있었으며 이는 VCAM-1 발현의 감소와 연관이 있었다. OLETF 쥐에서는 LETO 쥐와 비교했을 때 32주에 혈관내피세포 의존성 혈관확장능의 감소를 보였는데, LAB투여가 이를 일부 예방할 수 있음을 확인할 수 있었다.

결론적으로, LAB는 배양된 인간혈관 내피세포에서 고혈당에 의한 혈관내피세포 기능장애에 긍정적인 효과를 미쳤고 OLETF 쥐에서도

혈관 내피세포 기능장애를 예방할 수 있었다. 따라서, LAB는 향후 당뇨병관련 혈관 합병증의 치료제로서 사용될 수 있는 가능성이 있다고 할 수 있겠다.

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핵심 되는 말: magnesium lithospermate B, 혈관내피세포 기능장애, 당뇨병