A Master's Dissertation

Study of Endothelial Dependent Vasorelaxation Effects in Hypertension

Department of Veterinary Medicine
Graduate School, Jeju National University

Dong Geon Kim

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Dong Geon Kim
(Supervised by Professor Young Jae Lee)

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2010. 12.
This thesis has been examined and approved.

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Thesis director, Professor Chang-Hoon Han,
College of Veterinary Medicine

.....

Professor Young Jae Lee,
College of Veterinary Medicine

.....

Professor You Jin Jeon,
Department of Marine Life Science

.....
Date

Department of Veterinary Medicine
Graduate School, Jeju National University

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ABSTRACT

Hypertension and other risk factors for cardiovascular disease contribute to oxidative stress, which caused endothelial dysfunction. The most characteristic pathophysiological feature of endothelial dysfunction is a diminished nitric oxide (NO) bioavailability due to reduced endothelial NO synthase (eNOS) activity and/or increased metabolism through its interaction with superoxide anion (O_2^-) produced in the vascular wall. Therefore, vascular endothelial function is a strong marker for monitoring cardiovascular health on the basis that the endothelial dysfunction is the major factor of cardiovascular disease. Correction of endothelial dysfunction may be associated with reduced cardiovascular risk. This study was focused on endothelium may be a direct therapeutic target for treatment cardiovascular disease, especially hypertension. In this study was elucidated to the endothelial dependent anti-hypertensive effects of biotransformed Gwibi-tang and active compounds isolated from *Styela clava*

Part I. Endothelial dependent vasorelaxation by Biotransformed Gwibi-tang

Herbal medicines have been used to treat various diseases in Asian countries. One of these medicines, Gwibi-tang (GBT), is well known for its effect of treating cardiovascular disease. Biotransformed GBTs were produced by the fermentation using by *Lactobacillus sp.* to search metabolites on the basis of increased biological activity through its modified structure. Our hypothesis is that some metabolites in biotransformed GBT might improve anti-hypertensive effect through chemical modification. Anti-hypertensive effects of biotransformed GBT were observed by measuring angiotensin converting enzyme inhibitory activity, vasodilation and blood pressure. Biotransformed GBT, showed higher inhibitory

effect on angiotensin II-converting enzyme (ACE) activity than natural GBT. Above all GB166 (fermentation using by *Lactobacillus curvatus*) increased endothelial-dependent vasodilation. NO synthase (NOS) inhibition abolished GB166 mediated relaxation in aorta and NO synthesis and eNOS phosphorylation (Ser 1177) were increase eNOS compared with reduced $p47^{phox}$. Also, Systolic blood pressure was decreased after administration of GB166 (200 mg/kg, P.O.) in SHR. The results suggest that GB166 have improved anti-hypertensive effect for vasorelaxation by endothelial-dependent mechanism through chemical modification of natural compounds in GBT.

Part II. Vasorelaxation Effects of Partially Purified Fractions from *Styela clava*

The ascidian tunicate, classified as Urochordata animal, is considered to be native in the northwest Pacific region including Korea and widely distributed in northwestern Europe, North America. *Styela clava*, a species of tunicates, have been culturing at the southern coast of Korea and consuming as the foodstuffs. The anti-hypertensive activity of *Styela clava*, however, is currently unknown. The present study investigated anti-hypertension effects of active compound isolated from *Styela clava.* Anti-hypertensive effects of active compound isolated from *Styela clava* were observed by measuring indirect blood pressure and direct blood pressure using powerlab system. The study was conducted to examine the effects of angiotensin II converting enzyme (ACE) inhibitory activity and vasorelaxation response. Nitric oxide release and phosphorylation level of eNOS (Ser 1177) were measured in human vascular endothelial cells (EA.hy926 cell) cultured with active compound isolated from *Styela clava.* The active compound of isolated from *Styela clava* showed enhanced inhibitory effect on ACE activity. Vascular relaxation was improved by isolated active compound. Pre-incubation of aortic ring with $_{L}$-NAME (NOS inhibitor) abolished vasorelaxation, which suggest endothelium-dependent vasorelaxation. In

endothelial cells (EA.hy926 cell), NO synthesis was increase and eNOS phosphorylation (Ser 1177) was upregulated when the cells were cultured with active compound isolated from *Styela clava*. Systolic blood pressure was decreased after administration of compound isolated from *Styela clava* (50 mg/kg, I.V.) in SD-rat. The results show that active compounds of isolated from *Styela clava* could enhance eNOS phosphorylation and subsequent release of NO from endothelial cells. Therefore, the observed eNOS-inducing effects in response to isolated active compounds from *Styela clava* support a role of endothelial dependent mechanism. Overall results suggest that isolated active compound isolated from *Styela clava* has anti-hypertensive effect.

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INTRODUCTION

Hypertension is a slowly developing cardiovascular disorder which causes most of the morbidity and mortality in the elderly people (31). It is triggered by environmental influences such as obesity, salt intake, and lack of exercise (3). The specific genes responsible for hypertension have not been identified clearly (18). Untreated hypertensive causes heart failure, coronary artery disease, or acute renal failure. Patients have primary hypertension, which lead to many abnormalities of the cardiovascular smooth muscle and endothelium (3).

Vasodilator mechanisms may be characterized as endothelium-dependent or endothelium-independent (30). Hypertension is associated with impaired endothelium dependent vasorelaxation in response to stimuli (19). Such as acetylcholine and bradykinin, reduces classical vascular risk factors including hypertension (48). The vascular endothelium lies at the interface between the circulating blood cells and the vascular smooth muscle cells (63). The endothelium controls many important functions, including maintenance of blood circulation and fluidity as well as regulation of vascular tone, coagulation, and inflammatory responses (7, 20). Endothelium was synthesizes and release a broad spectrum of vasoactive substances, including NO, prostacyclin and endothelin-1 (2). The endothelium-derived relaxing factor is NO, which is known to be the primary endogenous vasodilator (60). Nitric oxide is generated from L-arginine by the calcium/calmodulin-dependent enzyme, nitric oxide synthase (NOS), in the site of vascular endothelium (39). After generation, NO diffuses out of the endothelial cells, and traverses membranes of vascular smooth muscle cells (VSMC) (45). Nitric oxide stimulates guanylate cyclase activity in the target cell, and increases cGMP level which results in muscle relaxation via decreased intracellular calcium (Ca^{2+}) (22). Nitric Oxide acts as a vasodilator, inhibits inflammation, and has anti-aggregate effects on platelets (39). Reduced NO has often been reported in the presence of endothelial

dysfunction, which results from reduced activity of eNOS or decreased NO production (5). Animal experiments and clinical data provide evidence that NO level is reduced by increased generation of ROS in the vessel wall (5, 25). Elevated concentrations of ROS such as superoxide anions and lipid peroxides may account for the impaired endothelium-dependent vasodilation in response to agonists or by scavenging endothelium-released NO (28).

Increased ROS production and a shift in balance from NO to ROS signaling represent common characteristics in cardiovascular disease (5, 28). The nitric oxide synthase (NOS) can produce large amount of O_2^- when deprived of their critical cofactor (tetrahydrobiopterin) or their substrate (L-arginine). In this state, referred to as NOS inactivation, electron flow through the enzyme results in reduction of molecular oxygen to form $\cdot O_2^-$ rather than formation of NO (40). A major product of reaction between superoxide and NO is peroxynitrite, which is a weaker vasodilator than NO, and this reaction markedly impairs the vasodilator capacity of NO (5).

Angiotensin II is a multifunctional hormone which is involved in blood pressure control, based on its role in renal salt and water regulation, as well as central nervous system and vascular smooth muscle tone (6, 67). In addition, angiotensin II stimulates the activity of membrane bound NADPH oxidase in vascular smooth muscle cells and endothelial cells (23, 72). Indeed, angiotensin II generates superoxide anion throughout the vessel, including the endothelium (42). Nitric oxide levels are significantly decreased due to superoxide production evoked by angiotensin II stimulation (50). Since angiotensin II is produced by angiotensin II converting enzyme (ACE), inhibition of ACE activity has clinical benefits to cardiovascular disease including congestive heart failure, stroke and hypertension (71). In hypertensive patients, inhibition of ACE activity restores endothelial function (56, 57).

In this study, the endothelial dependent vasorelaxation was analyzed using natural products *in vitro* and *in vivo*. The antihypertensive effects of biotransformed Gwibi-tang and partially purified fraction of *Styela clava* were observed.

PART I.

Endothelial Dependent Vaosrelaxation by Biotransformed Gwibi-tang

INTRODUCTION

Hypertension, one of most important cardiovascular disease, is associated with impaired endothelium dependent vasorelaxation in response to stimuli (19). Endothelium-dependent vasorelaxation is commonly associated with cardiovascular disease risk factors, including hypertension (33). The underlying causes of the impaired vasodilation are related to the synthesis or sensitivity to NO, or increased oxidative stress-mediated destruction of NO (11). Nitric oxide produced in the vascular endothelium sustains the vasodilator tone and inhibits platelet function, leukocyte adhesion, and smooth muscle cell proliferation (11). In keeping with these actions, diseases such as hypertension, hypercholesterolemia, and atherosclerosis share an endothelial dysfunction with abnormal synthesis of NO (16). Elevated NADPH oxidase-dependent oxidative stress impairs NO production and contributes to the endothelial dysfunction (53). Increased eNOS activity and/or antioxidant enzyme content of the vascular wall can reverse the endothelial dysfunction in hypertensive animals (10).

Herbal medicines have been used for traditional therapeutics to treat various diseases in Asian countries. Recently, herbal therapy has growing popularity due to its natural properties in Asia and Europe (70). Herbal medicines are known to be beneficial in the treatment of chronic cardiovascular disease, including hypertension, although the mechanism by which these herbal medicines function is not yet fully established (70).

Microbial-biotransformation has growing interest in the pharmaceutical industry, and the important field of biotechnology (58, 59). Especially, lactic acid bacteria are used widely in biotransformation technology (44). *Lactobacillus* sp. and *Bifidobacterium* sp., lactic acid bacteria of the predominant members of the intestinal microflora, are reported to exert beneficial effects including the activation of the immune system, reduction of serum cholesterol and the inhibition of growth of potential pathogens (27). Moreover, lactic acid,

one of the most important organic acids produced by lactic acid bacteria has a prime position due to its versatile application in foods, pharmaceuticals, and other chemical industries (51, 29). Several studies have shown that the fermentation of herbal extract with lactic acid bacteria improve the therapeutic benefits (68).

In this study, the endothelial dependent vasorelaxation was analyzed *in vitro* and *in vivo* studies. The antihypertensive effects of biotransformed Gwibi-tang produced by the fermentation using a series of *Lactobacillus sp.* and *Bifidobacterium* sp. were observed.

MATERIALS AND METHODS

Sample

Gwibi-tang, Korea traditional herbal medicine and biotransformed Gwibi-tang (Table 1) supplied form Korea institute of oriental medicine

Angiotensin converting enzyme inhibitory activity

The ACE inhibitory activity assay was performed using ACE inhibitory activity assay kit (ACE-kit WST, DOJINDO, USA). This kit is used for the determination of ACE (angiotensin-converting enzyme) inhibition activity. ACE inhibition Assay Kit enzymatically detects 3-Hydroxybutyric acid (3HB) which is made from 3-Hydryoxybutyryl-Gly-Gly-Gly (3HB-GGG). Using a 96-well format, it is possible to test multiple samples at one time.

Vascular reactivity

The descending thoracic aortas obtained from the experimental groups of rats were dissected and placed in an ice-cold and oxygenated Krebs solution containing (in mM): NaCl, 120; KCl, 4.75; Glucose, 6.4; $NaHCO_3$, 25; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 1.7. Rings of thoracic aorta (4 mm in length) were carefully excised and submerged in organ baths containing 10 ml of Krebs solutions of bathing medium at 37°C and continuously gassed with a carbogen mixture, 95% O2 and 5% CO2. The rings were mounted by means of two parallel triangle-shaped stainless-steel holder served as a anchor, while the other was connected to a force-displacement transducer (FT03, Grass, USA) to measure isometric

contractile force recorded by a physiograph recorder (PowerLab/800, USA). A basal tension of 1 g was applied. Each preparation was allowed to equilibrate for 60-90 min in Krebs solution prior to the initiation of the experimental procedures, and during this period, the incubation medium was changed every 15 min. After equilibration time, the aortic rings were exposed to norepinephrine (10^{-7} M). When the contraction had stabilized, acetylcholine (10^{-5} M) was added to test for the presence of the endothelium (Figure 1.). After equilibration time, the aortic rings were contracted by norepinephrine (10^{-7} M), and when the contractile response was stabilized (steady-state phase, 12-15 min), relaxation was evaluated by cumulative addition of sample (from 0.135 to 3 mg/ml). Cumulative concentration-response curves were also performed with in the presence and absence of $_{L}$-NAME (100 μmol/L) or endothelium – denuded artery segments. $_{L}$-NAME was preincubated for 30 minutes before experiments. The degrees of precontraction of artery segments with all of the treatments were similar to the control. Relaxation responses to samples were plotted as a percentage of relaxation from the maximum contraction.

A B

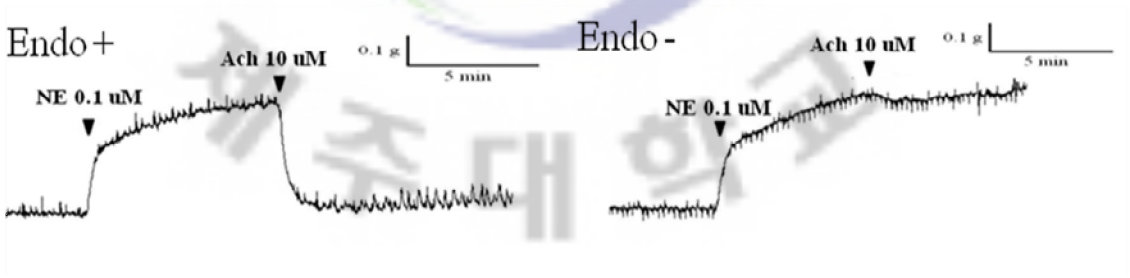


Figure 1. Vasorelaxation effect of acetylcholine on endothelial-intact (A) or -denuded (B) aorta contracted with norepinephrine.

Measurement of NOx released from arteries

Ring of thoracic arteries, approximately 5 mm in length, were cut from the arteries harvested as described above. The rings were rinsed several times in sterile ice- cold PBS with 1% antibiotic-antimycotic and placed in the walls of 24 well culture plates. Vessels rings from each artery were randomly divided in to either controls with 1 ml DMEM containing 1% antibiotic / antimycotic and 100 $\mu\ell$ PSB, GBcon, or GB166 treated with 1 ml DMEM containing 1% antibiotic / antimycotic. Vessel rings were cultured for 1 h in a cell culture incubator maintained a 37°C and 5% CO_2. NO metabolites (NOx; nitrite and nitrate) in media were measured by reduction of nitrate by vanadium (III) and detection with Griess reagents (41). After loading with diluted serum samples (100 $\mu\ell$) in 96-well microtiter plate, addition of VCl_3 (100 $\mu\ell$) to each well was rapidly followed by addition of the Griess reagents, 2% sulfanilamide (SULF; 50 $\mu\ell$) and 0.1% N-(1-naphthyl) ethlenediamine (NEDD; 50 $\mu\ell$). After incubation at 37°C, and then read absorbance at 570nm. And the results were expressed as μM of NOx.

Animals

All animal procedures were carried out in accordance with the National Institute of *Health Guide for the care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Utilization Committee for Veterinary Medicine of Jeju National University

11-week-old, male SHR and WKY rats were purchased from Japan SLC. Inc. (Shizuoka, Japan). All rats were maintained at 24 ± 1°C with 12-hour light/dark cycle and were given standard rat chow and water *ad libitum*. SHR were randomly divided into fourth groups. One group of SHR was given to PBS (P.O) as a control group (SHR), the second and

Third group of SHR were given GB original (GBcon, 200 mg/kg/day, P.O.) and biotransformed GB (GB166, 200 mg/kg/day, P.O.) The fourth group received Amlodipine (Am, 30 mg/kg/day, P.O.) and WKY rats served as a nomortensive control rats

Blood pressure measurements

Systolic blood pressure was measured in conscious rats by tail - cuff plethysmography. At least seven determinations were made in every session and the mean of the lowest three values within 5 mmHg was taken as the SBP. SBP was recorded with a physiograph recorder (powerLab 2/25, AD instruments, Colorado springs, USA) in conscious rats. The heart rate was measured by using a Chart 7 for Windows program (AD instruments, Colorado springs, USA) triggered by pulse wave level. At the end of experiments, direct blood pressure was measured in all animals. For this purpose, the rats were anesthetized with Zolietil50 (50 mg/kg, Virbac, Carros cedex, France) A polyethylene catheter (PE-50) containing 50 U heparin in isotonic, sterile 0.9% NaCl solution was inserted into the right carotid artery for intra-arterial blood pressure and heart rate measurement in conscious rat.

Organ weight indices

After the end of the experiment, the animals were euthanized with carbon dioxide, the blood was collected from the abdominal vein and then the heart and kidneys were excised, cleaned and weighed. The weight index of the heart and the kidney were calculated dividing each organ weight by the body weight (BW).

Measurements of NOx in serum

The serum level of NO metabolites (NOx; nitrite and nitrate) was measured by reduction of nitrate by vanadium (III) and detection with Griess reagents (41). After loading in 96-well microtiter plate with diluted serum samples (100 ㎕), addition of VCl_3 (100 ㎕) to each well was rapidly followed by addition of the Griess reagents, 2% sulfanilamide (SULF; 50 ㎕) and 0.1% N-(1-naphthyl)ethlenediamine (NEDD; 50 ㎕). After incubation at 37°C, and then read absorbance at 570nm. And the results were expressed as μM of NOx.

Western blot analysis

Frozen aortas were homogenized in homogenization buffer (50 mM Tris-HCl, pH 7.5, 150 mM Sodium chloride, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 5 ㎕/㎖ aprotinin, 5 ㎕/㎖ leupeptin, 1 mM DTT, 1 mM PMSF) with a glass pestle. The homogenates were centrifuged at 12,000 × g at 4°C for 20 min and the supernatants were then stored at -80°C until used. The protein concentration was measured using the Bradford method (4). Aliquots of the homogenates (40 ㎕ of protein for aorta) were resolved on 8% SDS-polyacrylamide gels for the detections of eNOS, eNOS phosphorylation and iNOS and 10% SDS-polyacrylamide gels for the detections of $p47^{phox}$ and α-actin proteins, and transferred to a polyvinylidene fluoride (PVDF) membrane (BIO-RAD, Hercules, CA) with a glycine transfer buffer [192 mM glycine, 25 mM Tris-HCl (pH 8.8), 20% MeOH (v/v)]. After blocking the nonspecific site with 5% nonfat dried milk, the membrane was then incubated with specific mouse monoclonal anti-eNOS and anti-iNOS (1:500, BD Transduction Laboratories, USA), ant- $p47^{phox}$ (1:500, Santa Cruz), anti- α-actin (1:5000, Santa Cruz) and specific rabbit polyclonal anti-phospho-eNOS (1:500, Cell

signaling, USA) as primary antibodies at 4℃ overnight. The membrane was further incubated for 30 min with a secondary peroxidase-conjugated IgG (1:5000, Santa Cruz) to mouse and rabbit. The immunoactive protein were detected using an enhanced chemiluminescence (ECL) western blotting detection kit (Amersharm-pharmacia Biotech)

Cell culture

Human endothelial cell line EA.hy926 cells were used transformed human endothelial cell line EA.hy926 cell was kindly provided by Dr. C. Edgell (University of North Carolina at Chapel Hill, NC). Cells were cultured at 37 °C in Dulbecco modified eagel medium (Invitrogen, Carlsbad, CA, USA) supplement with 10% fetal bovine serum (FBS) and antibiotics (penicillin G and stereptomycin).

Immunofluroescence Analysis

EA.hy926 cells were seeded on chamber slide (Lab-Tek) and grown to 80-90% confluence. Cells were fixed using cold acetone and permeabilized with 0.01% Triton X-100. After blocking the nonspecific site with 5% Bovine serum albumin, the chamber was then incubated with specific rabbit polyclonal anti-phospho-eNOS (1:100, Cell signaling, USA) at 2 hour. Secondary antibody was Alexa 488 (Molecular probes, goat anti-rabbit conjugated IgG 1 : 400, Invitrogen, USA) for 30min. Nuclei were visualized using 1.5 uM propidium iodide (SIGMA, USA).

High performance liquid chromatography (HPLC) analysis

Gwibi-tang separation was performed using a reverse-phase HPLC system consisting of an HPLC pump (LPG 3x00). Biotransformed samples were monitored at 250 nm. The major compounds of Gwibi-tang (decursin, decursinol, glycyrrhizin, 6-gingerol, costunolide, swertisin) were identified by retention time which were compared between pre- and post biotransformation of Gwibi-tang. The concentration of decursin, decursinol, glycyrrhizin, 6-gingerol, costunolide, and swertisin were determined with a system consisting of pump (LPG 3x100), a diode array UV/VIS detector (DAD-3000(RS)), a column oven (TCC-3000SD), and an autosampler (ACC-3000). Dionex C_{18} column (SHISHEDO C_{18} 5 μm, 4.6 I.D. × 250 mm) was used in this study. Mobile phase maintained 0.1 % TFA (Trifluoroacetic acid) water (A) / Acetonitrile (B) with a flow rate of l ml/min. Each sample was analyzed in triplicate. For the analysis of herbal medicine compared major compound using by Standard (decursin, decursinol, glycyrrhizin, 6-gingerol, costunolide, swertisin) were purchased form Sigma.

Statistics

Results were expressed as means ± S.E.M. The statistical significance of difference between the groups were determined using one-way analysis of variance (ANOVA) followed by the Duncan test. P values lower than 0.05 were considered signification.

RESULTS

Effects of GB166 on Vasorelaxations

Effects of GB166 on vasorelaxation was observed using rat thoracic aorta prepared with or without endothelium to see whether the reduction of blood pressure is related to the improvement of endothelium-dependent vasorelaxation by GB166. The rat aorta ring with endothelium was relaxed by biotranformed Gwibi-tang (GB) than original Gwibi-tang (GBcon). In particular, GB166 (1 mg/ml) was significantly enhanced vascular relaxation (maximum relaxation of 20.29%) than GBcon (Figure 2.). Vasorelaxation effect of GB166 was increased in its concentration-dependent manner (0.187 to 3 mg/ml) with the maximum relaxation of 87.01 ± 5% at concentration of 3 mg/ml in aortic rings prepared with endothelium (Figure 3A, 3C). Without endothelium, however, the relaxation effect of GB166 was reduced (maximum relaxation of 44.53 ± 6.9%) at concentration of 3 mg/ml in aortic rings (Figure 3B, 3D). $_{L}$-NAME (100 uM) attenuated the GB166 induced relaxation by 59 ± 3.5% in the endothelium-intact aorta, suggesting the role of NO in the endothelial-dependent vasorelaxation (Figure 3E). The results suggest that GB166 improves vasorelaxatoin by producing NO in endothelium of aorta.

Angiotensin inhibitory activity

To observe the effect of GB166 on angiotensin II converting enzyme (ACE), which induces endothelial dysfunction, ACE inhibitory activity of GB166 was measured. ACE inhibitory activities were increased in a dose-dependent manner of GB166 (Figure 4). Interestingly, GB166 exhibited higher ACE inhibitory activity (IC50 of 1.01mg/ml) than

GBcon (IC50 of 1.73 mg/ml). The result indicates that biotransformation improves ACE inhibitory activity of GB.

Effects of GB166 on NO levels in artery

To observe the effects of GB166 on NO production, rat thoracic aorta was prepared with endothelium, was incubated in media, and the levels of NO released were measured in the presence or absence of GB166. As shown in Figure 5, the levels of NO released in the incubation media of aortic rings were increased by GB166 treatment in a concentration dependent manner (0.75 – 3 mg/ml). In contrast, NO production by GB166 was not observed in aorta prepared without endothelium (data not shown). The result suggests that GB166 directly affects NO production in endothelium of aorta.

Effects of GB166 on blood pressure

Basal arterial blood pressure and heart rate were observed in SD rats after injection of GB166. As shown in Figure 6, injection of GB166 (100 mg/kg) significantly reduced both mean arterial blood pressure and heart rate. Systolic blood pressure was measured in SHR by using tail cuff method after administration of GB166. GB166 reduced blood pressure in 6 hrs after administration, whereas Amlodipine reduces blood pressure in 2 hrs after administration (Figure 7). Long term experiment was carried out to observe the change of blood pressure in SHR treated with GB166 and Amlodipine**.** At the beginning of the study (3-month old rat), SBP in SHR (200 ± 2 mmHg) was already higher than that observed in WKY rats (120 ± 2 mmHg) and these differences persisted as time progressed ($p < 0.05$). Even untreated SHR group showed small change of blood pressure over the period of experiment, GB166 or Amlodipine treated group showed significant reduction in SBP values,

which persisted until the end of experiment ($p < 0.05$, Figure 8.). Overall results suggest that GB166 decreases both blood pressure and heart rate *in vivo* system, SHR.

Effects of GB166 on organ hypertrophy

The hypertrophic changes in heart and kidney by high blood pressure were observed in SHR. The weight of heart in SHR was significantly greater than that in WKY. The weights of both heart and kidney were slightly reduced in SHR treated with GB166 and Amlodipine (Figure 10). The result suggests that hypertrophy of organs were attenuated by reducing blood pressure after treatment of GB166 and Amlodipine in SHR.

Effects of GB166 on eNOS phosphorlyation and $p47^{phox}$ expression

Since GB166 enhanced NO production from aorta, the study was further scrutinized the effect of GB166 on NO synthase activation. Based on Western blot analysis, the treatments of GB166 and Amlodipine markedly enhanced the eNOS phosphorylation in aorta of SHR (Figure 11). Even compared with WKY rat, the untreated SHR exhibited a reduced eNOS phosphorylation in aorta**.** Effects of GB166 on $p47^{phox}$ expression were observed to see whether improved endothelial dysfunction by GB166 is mediated by reduced vascular ROS production in SHR. Compared with WKY, SHR produced higher expression level of $p47^{phox}$ in vascular wall. The treatment with either GB166 or Amlodipine significantly decreased $p47^{phox}$ expression level (Figure 11). The results suggest that improved endothelial dysfunction by GB166 or Amlodipine result from decreased NADPH oxidase activity and increased eNOS activity.

Effects of GB166 on eNOS phosphorlyation level in EA.hy926 cell

Since GB166 enhanced NO production in aorta, the present study was further investigated the effect of GB166 on NO synthase activation. Based on immunofluorescence analysis, GB166 markedly increased eNOS phosphorylation (Ser 1177) level in endothelial cells whereas no significant change of eNOS phosphorylation was observed in cells treated with GBcon (Figure 13). These data showed that enhanced NO production might result from increased eNOS phosphorlyation by GB166.

High performance liquid chromatography (HPLC) analysis

This study was aimed to demonstrate that the enhanced effects of biotransformed herbal medicine. Biotransformed Gwibi-tang was produced by the fermentation using by *Lactobacillus sp.* to search metabolites on the basis of increased biological activity through its modified structure. Based on HPLC analysis, decursin, decursinol, glycyrrhizin, 6-gingerol, costunolide, and swertisin were detected in GBcon (figure 13). In biotransformed Gwibi-tang, however, only decursinol, glycyrrhizin, and decursin, were detected. Especially, decursinol (9%), glycyrrhizin (7.57%), and decursin (6.9%) were decreased in GB166 compared with GBcon (Table. 2). The result shows that biotransformation, by using *Lactobacillus sp.*, changed the compositions of original Gwibi-tang.

Table 1. List of *lactobacillus sp*. used for biotransformation of Gwibi-tang

Sample	No.	Strain
GB	original	
GB	con	Gwibi-tang autoclave
GB	127	*Lactobacillus casei*
GB	129	*Lactobacillus casei*
GB	144	*Lactobacillus plantarum*
GB	164	*Lactobacillus fermentum*
GB	166	*Lactobacillus curvatus*
GB	402	*Lactobacillus plantarum*
GB	442	*Lactobacillus delbrueckii*
GB	693	*Lactobacillus casei*
GB	744	*Bifidobacterium breve*
GB	3163	*Bifidobacterium gasseri*

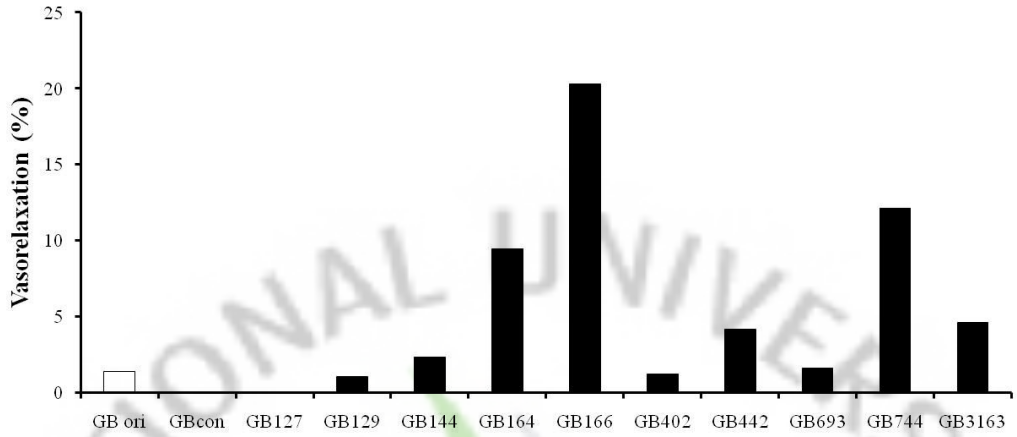


Figure 2. Vasorelaxation effects of biotransformed Gwibi-tang fermented by different *lactobacillus sp.*. Vasorelaxation effects of biotransformed Gwibi-tang (1 mg/ml) were observed using aortic rings preconstricted with norepinephrine (0.1 uM).

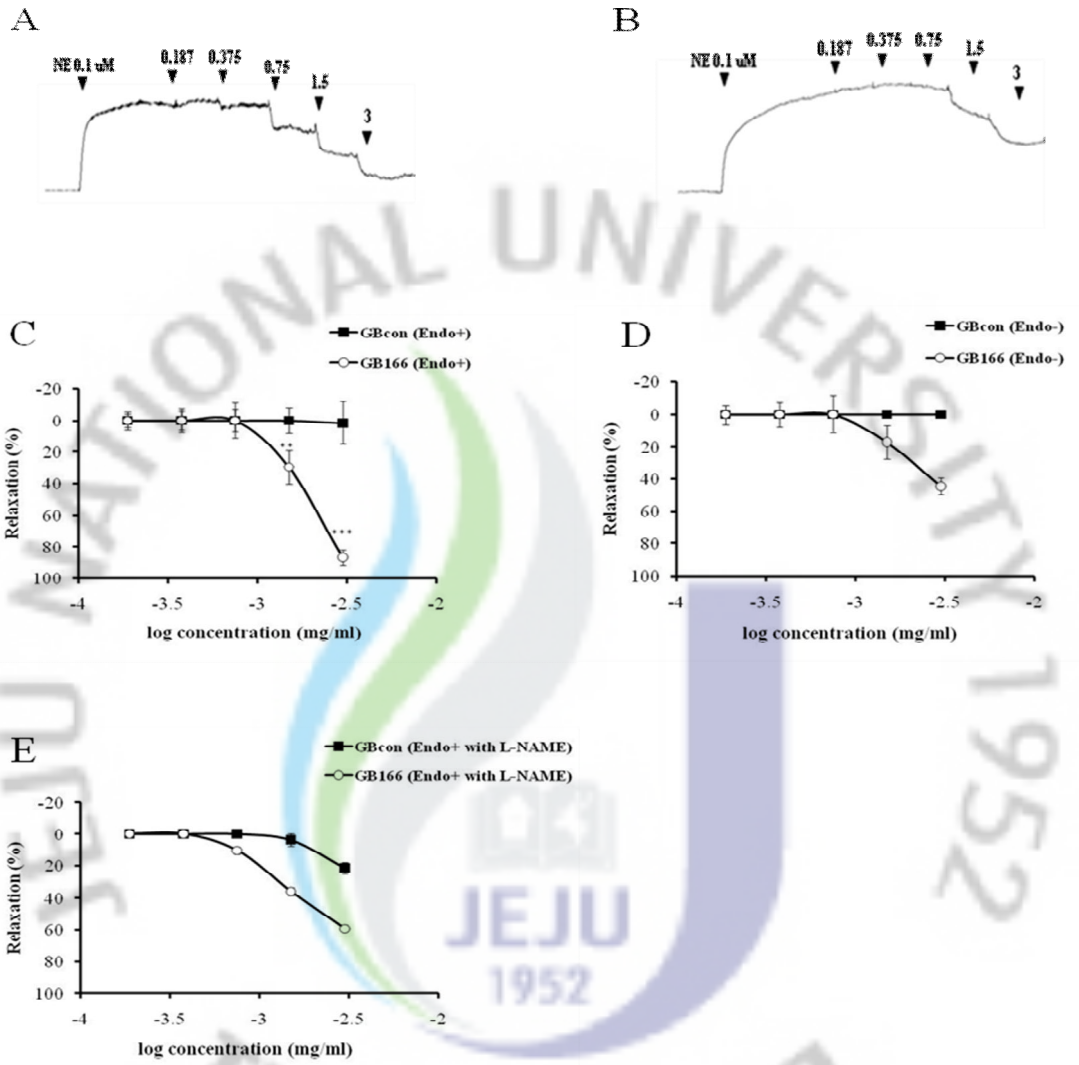


Figure 3. Endothelial-dependent vasorelaxation by GB166. Rat thoracic aortic rings with endothelium (A, C), without endothelium (B, D), and with endothelium plus L-NAME (100 uM) preincubation (E) were prepared, and vasorelaxation effects of GB166 (from 0.187 to 3.0 mg/ml) were observed. Data are means ± S.E.M. *$p < 0.05$ and **$p < 0.01$ compared with GBcon.

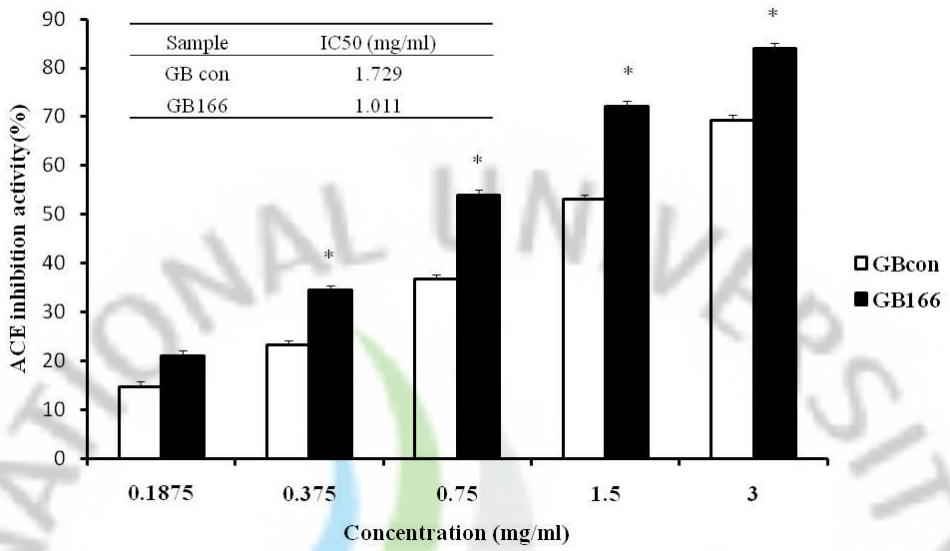


Figure 4. Angiotensin-II converting enzyme (ACE) inhibitory effects GB166. The effects of GB166 ranged from 0.187 mg/ml to 3 mg/ml were observed. Data are means ± S.E.M. *p < 0.05 compared with GBcon.

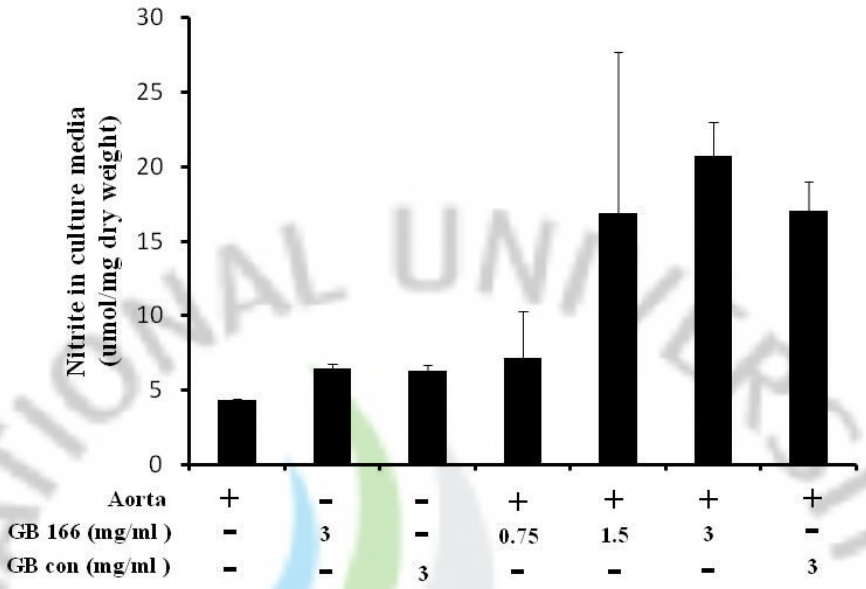


Figure 5. Effects of GB166 on nitric oxide (NO) levels released from thoracic artery. The NO concentration was determined by detection of total nitrite and nitrate released from isolated thoracic artery treated with GB166 and GBcon in media.

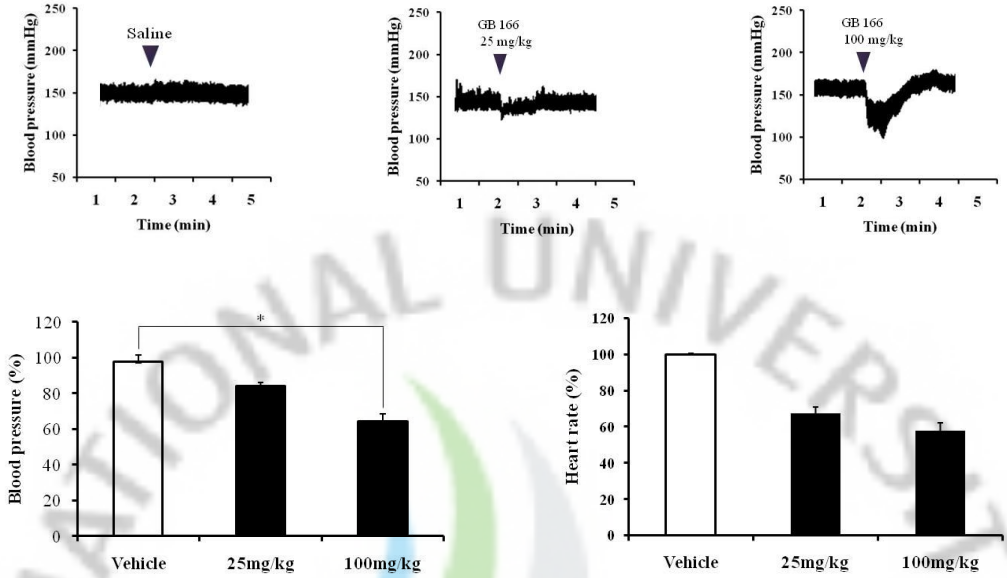


Figure 6. Effects of GB166 on direct blood pressure in SD rat. Basal arterial blood pressure (MAP) and basal heart rate (HR) were observed in SD rats as described in Materials and Methods. Data are means ± S.E.M. *p < 0.05 compared with control.

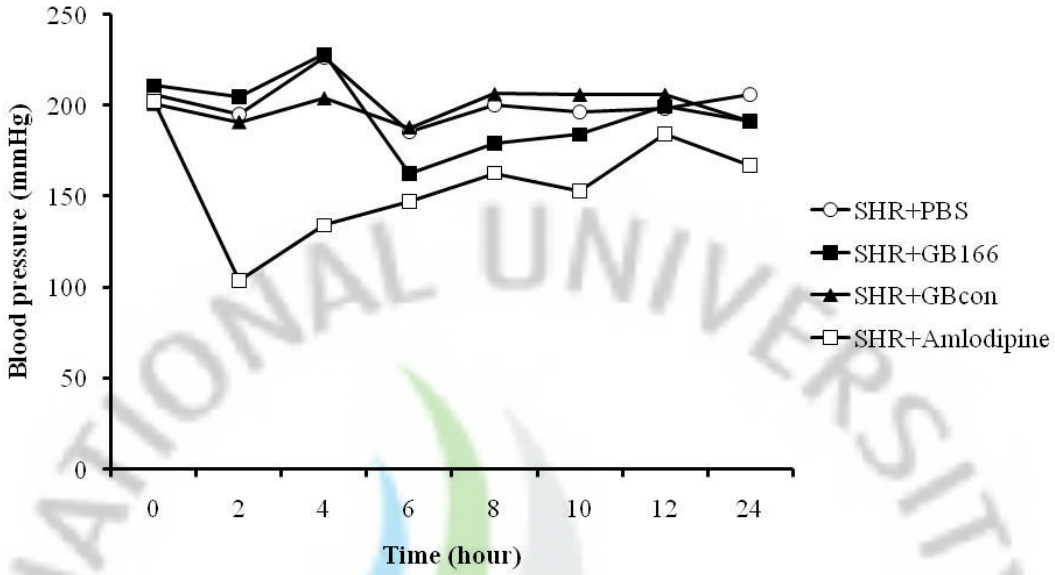


Figure 7. Effects of GB166 on blood pressure in SHR (for 24 hours). Systolic blood pressures were measured by tail cuff plethysmography in SHR administered with PBS (○), GB166 200 mg/kg (■), GBcon 200 mg/kg (▲), and Amlodipine 3 mg/kg (□). Blood pressure Measured was for 24 hours after single administration (P.O.).

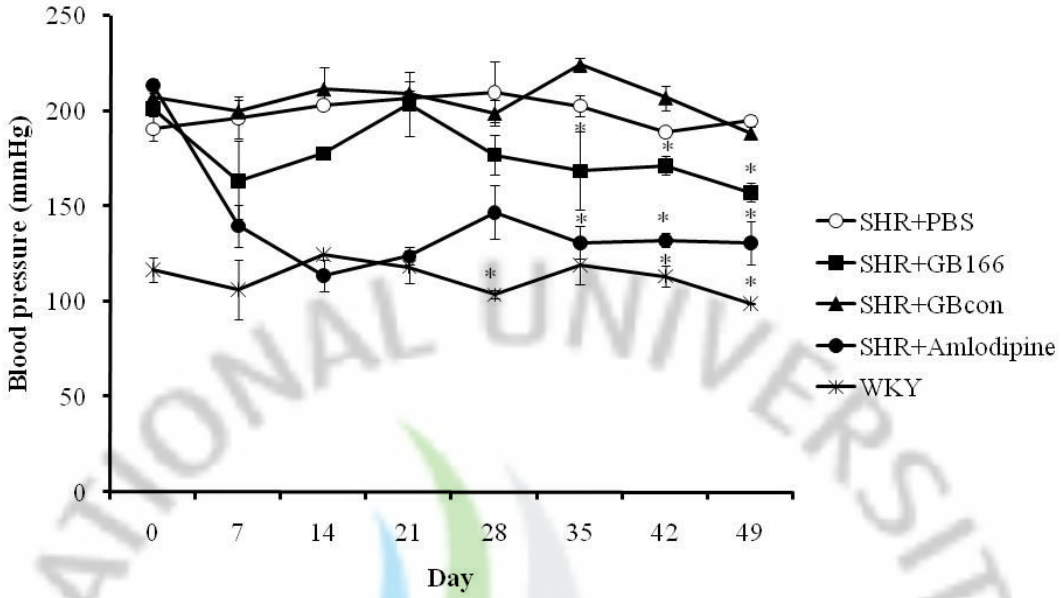


Figure 8. Effects of GB166 on blood pressure in SHR. Systolic blood pressures were measured by tail cuff plethysmography in SHR administered with PBS (○), GB166 200 mg/kg (■), GBcon 200 mg/kg (▲), and Amlodipine 3 mg/kg (□). Wister Kyoto rat (WKY) was used for control (×). Samples were administrated (P.O.) every day for 2 month in SHR/WKY and blood pressures were measured at once a week. Data are means ± S.E.M. *p < 0.05 and **p < 0.01 compared with GBcon.

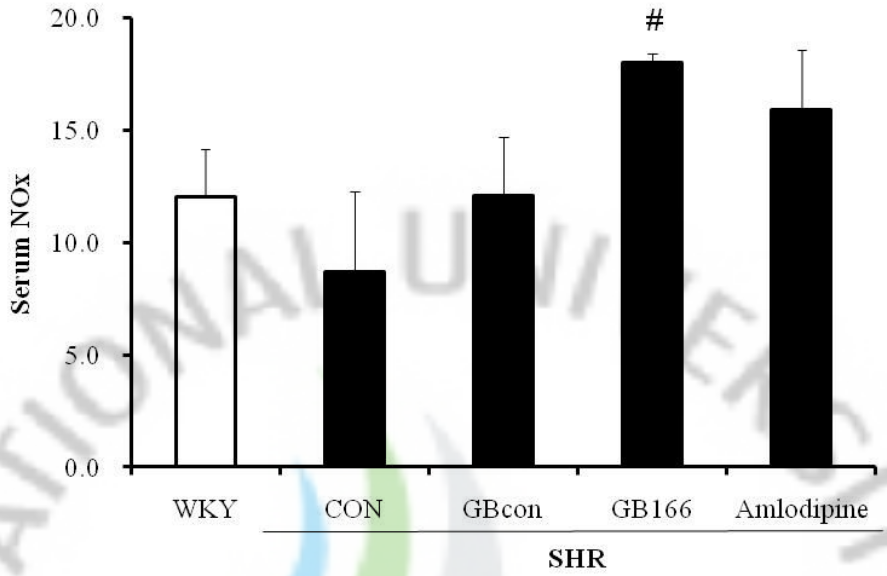


Figure 9. Effects of GB166 on NOx levels in Serum of SHR. Serum NOx levels were measured in each experimental group. As described in Materials and Methods. Data are means ± S.E.M. #$p < 0.05$ compared with SHR.

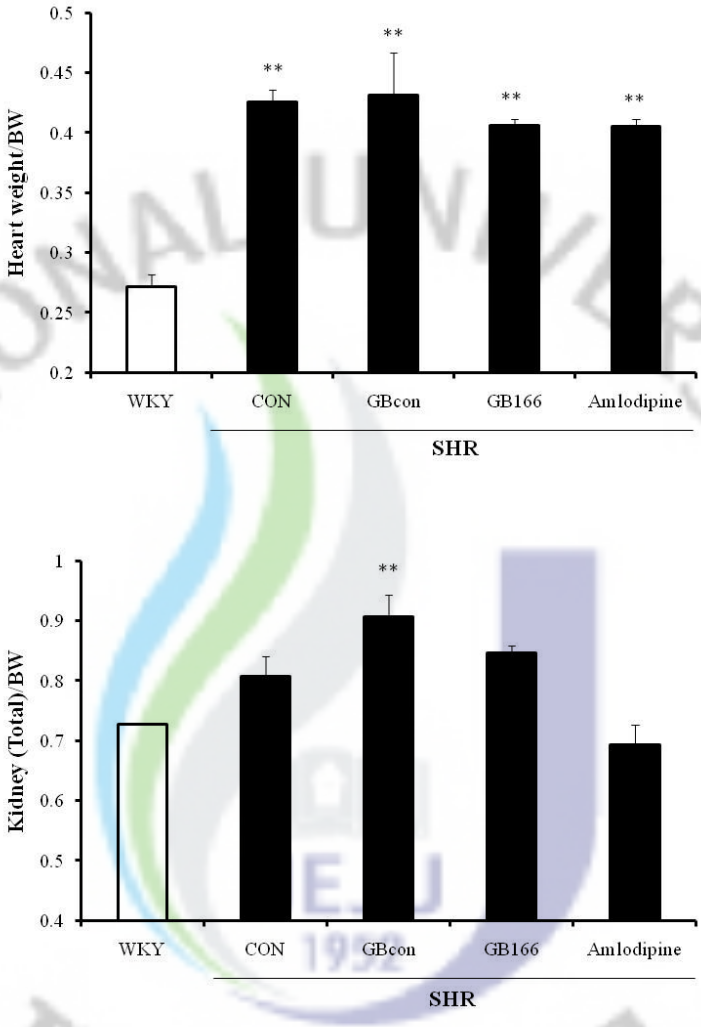


Figure 10. Effects of GB166 on the organ hypertrophy. Heart (A) and kidney (B) weights were measured and compared among the groups. Data are means ± S.E.M. *p < 0.05 and **p < 0.01 compared with WKY.

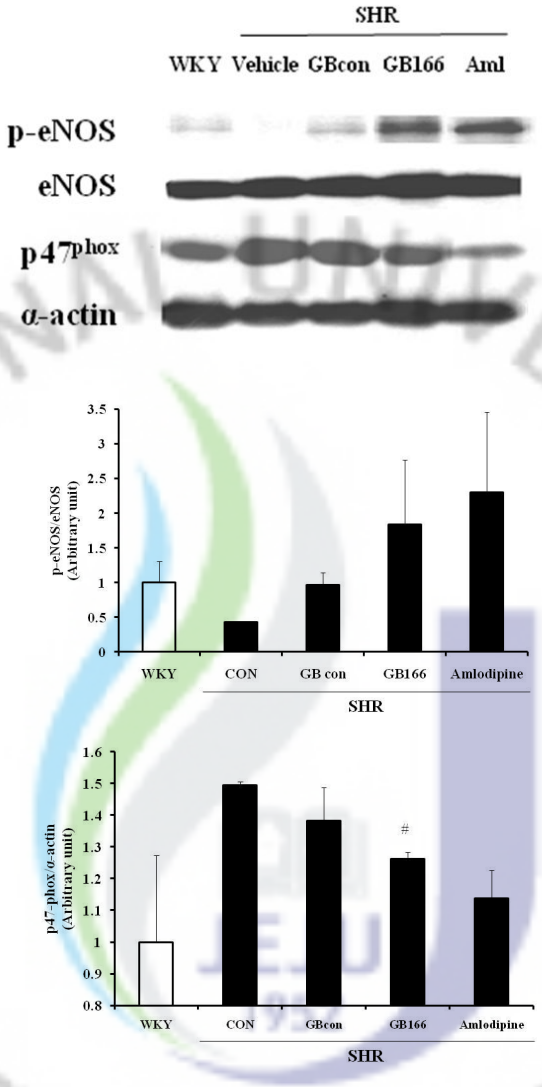


Figure 11. Effects of GB166 on eNOS phosphorylation and p47^{phox} expression in aorta of SHR. The eNOS phosphorylation level and p47^{phox} expression were determined by Western blot analysis in aorta of SHR on last day of the experiment after treatment for 2 month. The values from the densitometric analysis were calculated in relation to the concentration β-actin. Data are means ± S.E.M. #$p < 0.05$ compared with SHR.

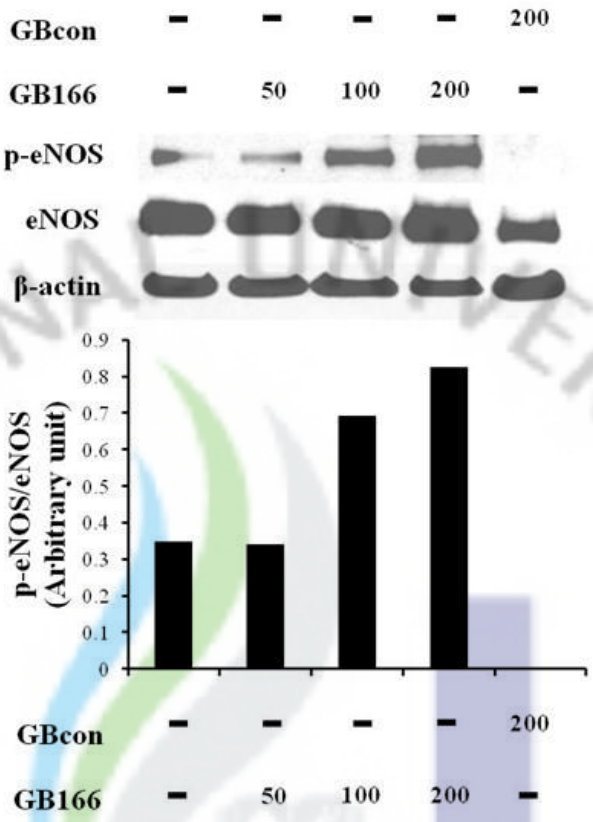


Figure 12. Effects of GB166 on a eNOS phosphorylation in endothelial cells (EA.hy926 cell). The eNOS phosphorylation level was determined by Western blot analysis in endothelial cells treated with different dose of GB166 (50 – 200 ug/ml). The values from the densitometric analysis were calculated in relation to the concentration β-actin.

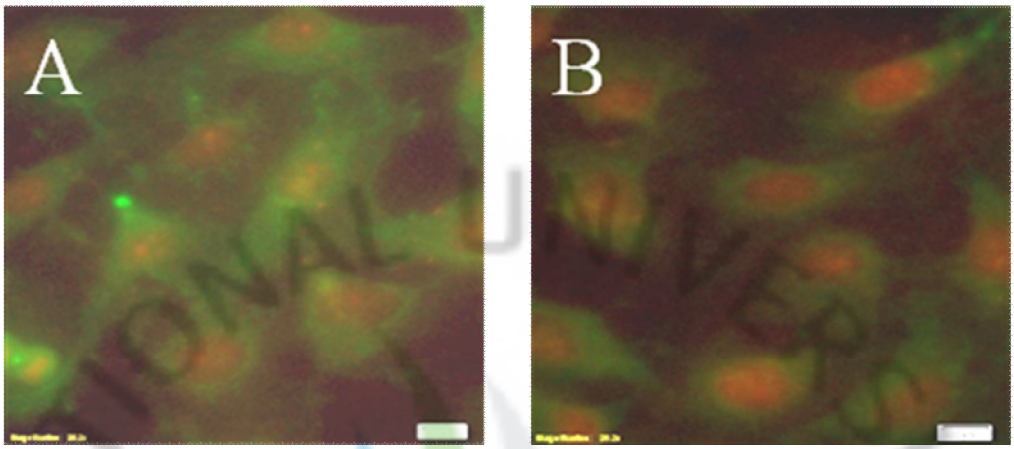
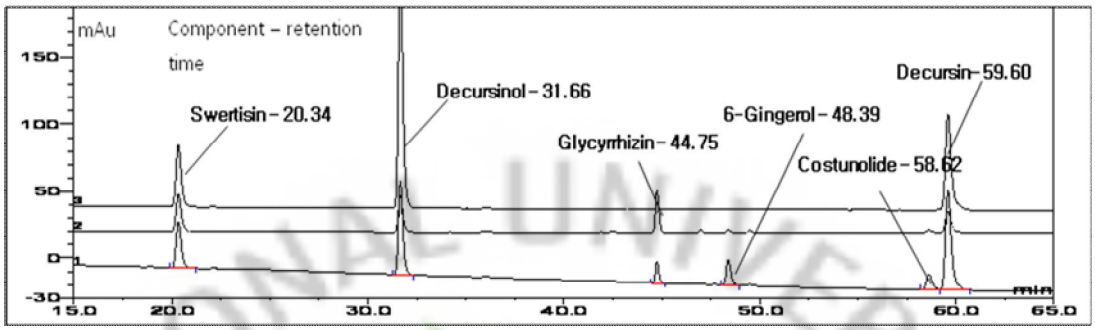


Figure 13. Effects of GB166 on eNOS phosphorylation (Ser 1177) in EA.hy926 cell. EA.hy926 cells were treated with GB166 (A) and GBcon (B) for 24 hrs, and were fixed with acetone. Anti-phosphos eNOS (Ser 1177) labled with fluorescence is shown in green, and DNA stained with PI is shown in red.

(A) Standard mixture



(B) GB con

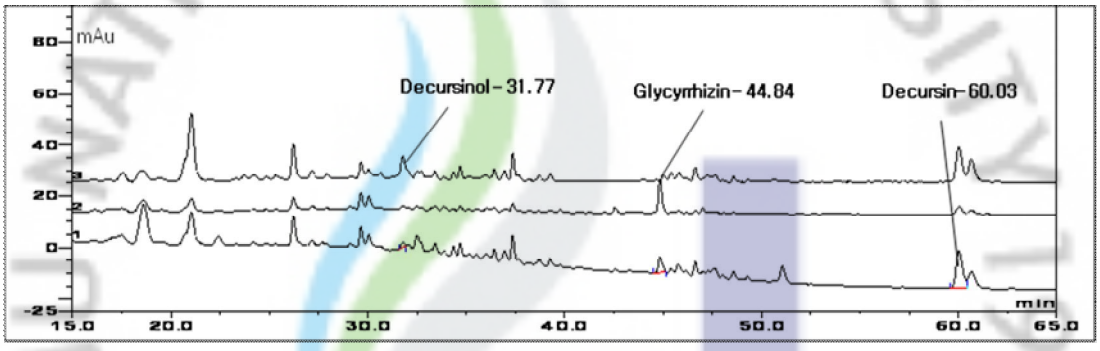


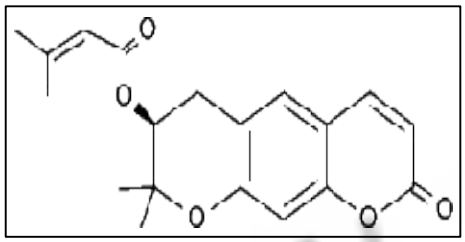
Figure 14. HPLC chromatogram of standard compound (A) and Gwibi-tang control (B).

Standard mixture (Decurisin, dicurisinol, glvyrrhizin, 6-gingerol, costunolide and swertisin) (A) and the compounds in GB control (B) were separated on HPLC, and were monitored at 250 nm.

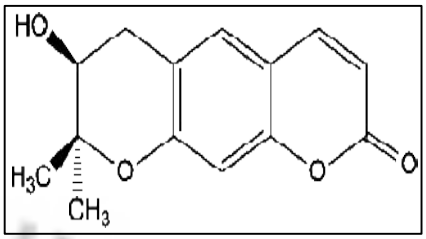
Table 2. Change of the compounds concentration in biotransformed Gwibi-tang

Sample	Decursinol		Glycyrrhizin		Decursin	
	ug/mg	%	ug/mg	%	ug/mg	%
GB control	0.173		1.646		0.697	
GB 127	0.165	-4.62	1.541	-6.34	0.656	-5.84
GB 129	0.166	-3.97	1.503	-8.66	0.639	-8.23
GB 144	0.166	-4.17	1.537	-6.62	0.653	-6.29
GB 164	0.177	2.61	1.56	-5.19	0.666	-4.47
GB 166	0.157	-9	1.521	-7.57	0.649	-6.9
GB 402	0.173	0.08	1.58	-4	0.669	-4.01
GB 442	0.186	7.57	1.551	-5.76	0.667	-4.32
GB 693	0.205	18.56	1.512	-8.12	0.642	-7.81
GB 744	0.174	0.86	1.523	-7.44	0.646	-7.26
GB 3163	0.161	-6.63	1.535	-6.7	0.662	-5

A



B



C

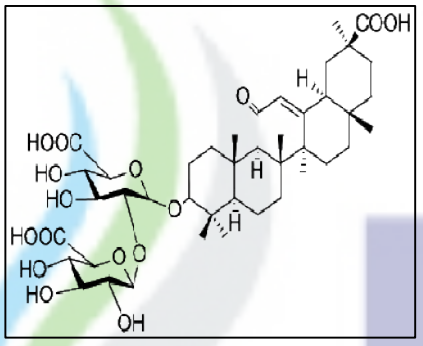


Figure 15. The structure of compounds isolated from Gwibi-tang

A : Decursin

B : Decursinol

C : Glycyrrhizin

DISCUSSION

In the present study, biotransformed Gwibi-tang fermented by using *Lactobacillus sp.* showed higher inhibitory effect on angiotensin II-converting enzyme (ACE) activity than original Gwibi-tang. Above all GB166 (fermented by using *Lactobacillus curvatus*) showed the highest endothelial-dependent vasorelaxation. In addition, the vasrelaxation effect of GB166 was significantly attenuated by NO synthase (NOS) inhibitor, $_{L}$-NAME, in aorta prepared with endothelium. Treatment of aorta with GB166 increased the release of NO from endothelial cell, which result from the enhancement of vascular eNOS phosphorylation. Moreover, the aorta isolated from GB166 treated group showed lower vascular $p47^{phox}$ expression as compared with the aorta isolated from GBcon group. Overall results suggest that biotransformation of herbal medicine GB166 would allow the development of new therapeutic drug for hypertension based on endothelial-dependent vasorelaxation mechanism.

In the present study, the effects of GB166 on vasorelaxation were observed using rat thoracic aorta prepared with or without endothelium. The endothelial dependent vasorelaxation activity of GB166 was reduced by removal of endothelial cells in aorta. Moreover, blockade of the NO synthase with $_{L}$-NAME casused a significant inhibition of the relaxant effect of GB166.

Vasorelaxation mechanisms may be characterized as either endothelium-dependent or -independent (30). Acetylcholine and shear stress are usually used for inducing endothelium-dependent vasorelaxation, whereas sodium nitroprusside (SNP) is used for inducing endothelium-independent vasorelaxation (EIDV) (45).

Nitric oxide is the endothelium-derived relaxing factor. Furchgott and Zawadzki (1980) showed that the normal relaxation response to acetylcholine was abolished when the endothelial lining was rubbed off (20). Palmer et al. (1987) identified the endothelium-

derived relaxation factor is NO, which is known to be the primary endogenous vasodilator (45). $_{L}$-NAME is a prodrug which has NO synthase inhibitory activity and is rapidly hydrolyzed in biological tissues (61). Nitric oxide is generated from L-arginine by the calcium/calmodulin-dependent enzyme, nitric oxide synthase (NOS), in the site of vascular endothelium (39). After synthesis, NO diffuses out of the endothelial cells, and traverses membranes of vascular smooth muscle cells (VSMC) (45). Nitric oxide stimulates guanylate cyclase activity in target cells, and increases cGMP level which results in muscle relaxation via decreased intracellular calcium (Ca^{2+}) (22). In this study, GB166 induced the endothelial-dependent vasorelaxation in aorta by increasing NO production which might result from enhanced eNOS phosphorylation (Ser1177) by GB166.

In the present study, biotransformed Gwibi-tang fermented by using *Lactobacillus sp.* showed higher inhibitory effect on angiotensin II-converting enzyme (ACE) activity than original Gwibi-tang. The previously studies have suggested that endothelial dysfunction may be therapeutically reversible (8), and ACE inhibitors and angiotensin receptor antagonist were found to improve flow-evoked endothelium-dependent vasorelaxation (9, 21, 47, 55). Angiotensin II has been implicated in the pathophysiology of vascular disease. Production of Ang II begins with conversion of angiotensinogen to angiotensin I by rennin, which is transformed to Angiontensin II by the catalyzing action of the ACE (50). Ang II decreases NO level by promoting oxidative stress (15, 50), increases ROS by stimulating NADPH oxidase (62), and promotes vascular inflammation (57). Angiotensin II can stimulate the vascular endothelial cells to increase the production of active oxygen, and increase the oxidative stress level in plasma (62). In hypertensive patients, interruption of the rennin-angiotensin system with ACEI of Angiotensin II receptor blockers restores endothelial function (56, 57). In this study, GB166 showed the ACE inhibitory effects which might be relevant to increase NO production and decrease superoxide generation by GB166.

The present study, treatment with either GB166 or Amlodipine induced significant

reduction in blood pressure in SHR. Moreover, Serum NO production was increased in GB166 treated groups. Treatment of SHR with either GB166 or Amlodipine increased eNOS phosphorylation levels, which might result in increased NO production in aorta. In addition, GB166 or Amlodipine treatment decreased the expression levels of $p47^{phox}$, which might result in reduced O_2^- generation in SHR aorta. The spontaneously hypertensive rat is commonly used as a model of impaired endothelium-dependent vaorelaxation (52). The basal levels of eNOS expression and phosphorylation of SHR were significantly lower than those of WKY, which is consistent with decreased endothelium-dependent relaxation and higher blood pressure levels in SHR (65).

After synthesis by phosphorylated eNOS, NO diffuses out of the endothelial cells, and traverses membranes of vascular smooth muscle cells (VSMC) (45). Nitric oxide stimulates guanylate cyclase activity in the target cell, and increases cGMP level which results in relaxation of smooth muscle via decreased intracellular calcium (Ca^{2+}) (22). Moreover, cGMP activates cGK-1, which mediates vascular relaxation via phosphorylation of several proteins regulating intracellular $Ca2^+$ - mobilization (49). Previous studies demonstrated that the pharmacological actions of the Ca^{2+} channel antagonists, Amlodipine, in eNOS/NO cascade system (54, 37). Triggering of eNOS/NO cascade system leads to vasorelaxation by decreasing intracellular concentration of Ca^{2+} in smooth muscle (37).

Based on previous study, the increased radical oxygen species levels are further enhanced by dephosphorylation of endothelial eNOS, which ceases to produce NO and instead switches to superoxide production (40). Superoxide anions (O_2^-) play a pivotal role by reacting with NO resulting in the formation of peroxynitrite ($ONOO^-$), and hence decreasing bioavailability of NO (5). The major enzymes for O_2^- generation in the vessel are NADPH oxidase (25, 62), Xanthin oxidase (34), and inactivated NOS (64). NADPH oxidase is a multi-subunit enzyme complex responsible for the monoelctronic reduction of oxygen to produce O_2^- (1). This enzyme complex is considered to be the breakdown of NO associated

with endothelial dysfunction in hypertension (24). Previous studies have suggested a pivotal role of the $p47^{phox}$ subunit of NADPH oxidase in the vascular oxidative stress and blood pressure (35, 38).

In this study, the effect of GB166 on the endothelial dependent vasorelaxation might result from increased NO bioavailability by enhanced eNOS/NO cascade system, and decreased O_2^- generation by reduced expression of NADPH oxidase subunit, $p47^{phox}$, in SHR aorta.

Biotransformed Gwibi-tang was produced by fermentation using lactic acid bacteria, *Lactobacillus* sp.. Based on HPLC analysis, the concentrations of several components (decursinol, decurisin and glycyrrhizin) were decreased in biotransformed Gwibi-tang as compared with original Gwibi-tang. In particular, biotransformed Gwibi-tang 166 (GB166) fermented by using *Lactobacillus curvatus* showed markedly increased antihypertensive effect than Gwibi-tang control (GBcon).

Microbial transformation is importance in correlation with the corresponding metabolism (biotransformation) and the structural modification of complex drug molecule which involve in stereoselective, regiospecific and selective conversions of natural drugs to their derivatives by microorganisms (66). The biotransfomed ginsenosides, which has improved pharmacological activity, can be produced via the hydrolysis of the sugar moieties in the major ginsenosides using microbial fermentation (46). Ko et al (2007) produced the biotransformed ginsenosides by using enzymatic hydrolysis prepared from the intestinal bacterial metabolite. Moreover, metabolites such as microbial exopolysaccharides synthesized by lactic acid bacteria showed enhanced productivities and advanced modification (13).

In this study, the structural modification (e.g. hydroxylation or carboxylation) of metabolites in GB166 might improve pharmacological activity of original Gwibi-tang. Even though HPLC analysis showed the changed concentrations of components (decursinol,

decurisin and glycyrrhizin) in GB166, the minor changes of chemical structure are hard to detect based on retention time of HPLC. In addition, we cannot exclude the possibility of some metabolites excreted from by *Lactobacillus curvatus* during the fermentation might affected increased biological activity of GB166. Further study should be investigated to evaluate active compound in biotransformed herbal medicine.

In this study, GB166 induced the endothelial-dependent vasorelaxation in aorta by increasing NO production which might result from enhanced eNOS phosphorylation (Ser1177). In addition, the ACE inhibitory effect of GB166 might increase NO production and decrease superoxide generation. The effect of GB166 on the endothelial dependent vasorelaxation might result from increased NO bioavailability by enhanced eNOS/NO cascade system, and decreased O_2^- generation by reduced expression of NADPH oxidase subunit, $p47^{phox}$, in SHR aorta. The structural changes (e.g. hydroxylation or carboxylation) of metabolites in GB166 might cause the increased pharmacological activity of original Gwibi-tang. However, we cannot exclude the possibility of some metabolites excreted from by *Lactobacillus curvatus* during the fermentation might affected increased biological activity of GB166. Further study should be investigated to evaluate active compound in biotransformed herbal medicine. Overall results suggest that the biotransformation of herbal medicine can be applied for the development of new therapeutic drugs.

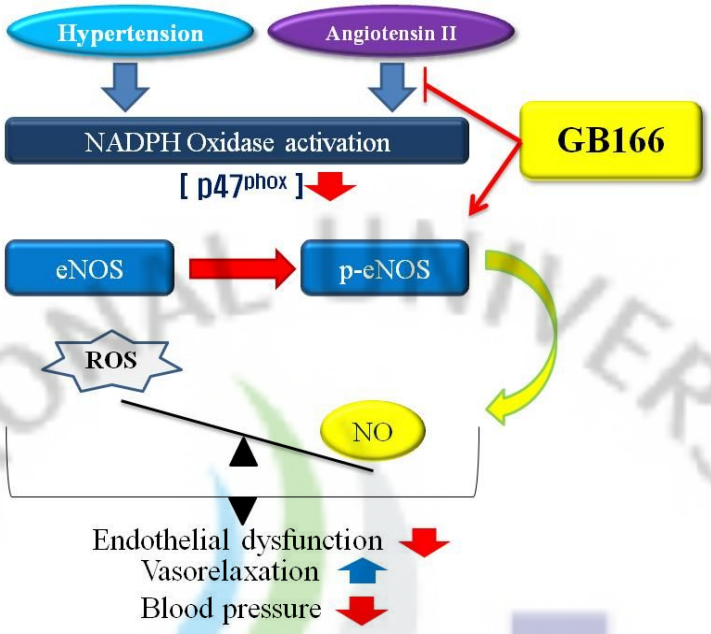


Figure 16. Proposed mechanism of endothelial-dependent vaorelaxation by GB166.

PART II.

Vasorelaxation Effects of

Partially Purified Fractions from *Styela clava*

INTRODUCTION

Vasodilator mechanisms may be characterized as endothelium-dependent or endothelium-independent (30). Moreover, Hypertension is associated with impaired endothelium dependent vasorelaxation in response to stimuli (19). Vasrelaxation effects were major mechanism of antihypertensive effects. Endothelium-dependent vasorelaxation induced by increased blood flow and receptor specific agonists, such as acetylcholine and bradykinin, are reduced in the presence of classical vascular risk factors including hypertension (48). Especially, the endothelium regulated tone at rest and during exercise, the thrombotic and adhesive properties of the vascular wall, the architecture of the vascular wall, and vascular wall, and vascular permeability. (48)

The vascular endothelium lies at the interface between the circulating blood cells and the vascular smooth muscle cells, responds to flow and shear stress and to vasoactive stimuli, and plays a crucial role in regulating blood flow and vascular tone (63). The endothelium is a continuous single layer of cells located between the wall of blood vessels and the blood stream (2). The functions of endothelium are numerous according to size and distribution of blood vessels (2). The endothelium controls many important functions, including maintenance of blood circulation and fluidity as well as regulation of vascular tone, coagulation, and inflammatory responses (7, 20). Endothelium was synthesizes and release a broad spectrum of vasoactive substances, including NO, prostacyclin and endothelin-1 (2). The endothelium is capable of producing a large variety of different molecules, as agonists or antagonists, therefore balancing the effects in both directions (7).

Nitric Oxide acts as a vasodilator, inhibits inflammation, and has anti-aggregant effects on platelets (39). Reduced NO has often been reported in the presence of impaired endothelial function, which results from reduced activity of eNOS or decreased NO

production (5). A number of model of endothelial dysfunction in experimental animals together with clinical data provide evidence that NO level is reduced by increased production of ROS in the vessel wall (5, 25). There are numerous evidences that increased oxidative stress is associated with endothelial dysfunction. Elevated concentrations of ROS such as superoxide anions and lipid peroxides may account for the impaired endothelium-dependent vasodilation in response to agonists or by scavenging endothelium-released NO (28)

The *ascidian tunicate*, classified as Urochordata animal, is widely distributed to the northwest Pacific region, northwestern Europe, and North America (12). *Styela clava*, a species of tunicates, have been culturing at the southern coast of Korea and consuming as the foodstuffs (17). Several studies reported that the effects of extract active compounds from *Styela clava* on anti-imflammaton and anti-bacterial (69, 36). However, investigated anti hypertensive effect were not clearly. In this study, the endothelial dependent vasorelaxation was analyzed *in vitro* and *in vivo* studies. The antihypertensive effects of partially purified fraction from *Styela clava* were observed.

MATERIALS AND METHODS

Isolation of compounds from *Styela clava*

Partially purified fraction from *Styela clava* samples were supplied from Prof. Yu Jin Jun, Department of Aquatic Life Medicine, Jeju National University, Korea. Extract of *Styela clava* using by enzyme hydrolysis (Protamex, NOVOZYME, USA). Enzyme hydrolysis extract were separated ultrafilteration (<5 KDa). Separated low molecular (< 5 KDa) extract was separated by using Sephadex G-25. Active compound was isolated first eluted fraction in molecular exclusion chromatography (SCF1)

Angiotensin converting enzyme inhibitory activity

The ACE inhibitory activity assay was performed using ACE inhibitory activity assay kit (ACE-kit wst, DOJINDO, USA). This kit is used for the determination of ACE (angiotensin-converting enzyme) inhibition activity. ACE inhibition Assay Kit enzymatically detects 3-Hydroxybutyric acid (3HB) which is made from 3-Hydryoxybutyryl-Gly-Gly-Gly (3HB-GGG). Using a 96-well format, it is possible to test multiple samples at one time.

Animals

All animal procedures were carried out in accordance with the National Institute of *Health Guide for the care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Utilization Committee for Veterinary Medicine of Jeju National University.

11-week-old, male SD rat were purchased from Japan SLC. Inc. (Shizuoka, Japan). All rats were maintained at 24 ± 1°C with 12-hour light/dark cycle and were given standard rat chow and water *ad libitum*.

Blood pressure measurements

Direct blood pressure was measured in all animals. For this purpose, the rats were anesthetized with Zolietil50 (50 mg/kg, Virbac, Carros cedex, France) A polyethylene catheter (PE-50) containing 50 U heparin in isotonic, sterile 0.9% NaCl solution was inserted into the right carotid artery for intra-arterial blood pressure and heart rate measurement in conscious rat. Mean direct arterial blood pressure was recorded after treatment active compound isolated from *Styela clava* (50 mg/ml, I.V.) with a physiograph recorder (PowerLab 2/25, AD instruments, Colorado springs, USA) in rats. The heart rate was measured by using a Chart 7 for Windows program (AD instruments, Colorado springs, USA) triggered by pulse wave level.

Vascular reactivity

The descending thoracic aortas obtained from the experimental groups of rats were dissected and placed in an ice-cold and oxygenated Krebs solution containing (in mM): NaCl, 120; KCl, 4.75; Glucose, 6.4; $NaHCO_3$, 25; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 1.7. Rings of thoracic aorta (4 mm in length) were carefully excised and submerged in organ baths containing 10 ml of Krebs solutions of bathing medium at 37 °C and continuosly gassed with a carbogen mixture, 95% O2 and 5% CO2. The rings were mounted by means of two parallel triangle-shaped stainless-steel holder served as a anchor, while the other was connected to a force-displacement transducer (FT03, Grass, USA) to measure isometric contractile force

recorded by a physiograph recorder (PowerLab/800, USA). A basal tension of 1 g was applied. Each preparation was allowed to equilibrate for 60-90 min in Krebs solution prior to the initiation of the experimental procedures, and during this period, the incubation medium was changed every 15 min. After equilibration time, the aortic rings were exposed to norepinephrine (10^{-7} M). When the contraction had stabilized, acetylcholine (10^{-5} M) was added to test for the presence of the endothelium. After equilibration time, the aortic rings were contracted by norepinephrine (10^{-7} M), and when the contractile response was stabilized (steady-state phase, 12-15 min), relaxation was evaluated by cumulative addition of sample (from 0.135 to 3 mg/ml). Cumulative concentration-response curves were also performed with in the presence and absence of $_{L}$-NAME (100 μmol/L) or endothelium – denuded artery segments. $_{L}$-NAME was preincubated for 30 minutes before experiments. The degrees of precontraction of artery segments with all of the treatments were similar to the control. Relaxation responses to samples were plotted as a percentage of relaxation from the maximum contraction.

Measurement of NOx released from arteries

Ring of thoracic arteries, approximately 5 mm in length, were cut from the arteries harvested as described above. The rings were rinsed several times in sterile ice- cold PBS with 1% antibiotic-antimycotic and placed in the walls of 24 well culture plates. Vessels rings from each artery were randomly divided in to either controls with 1 ml DMEM containing 1% antibiotic / antimycotic and 100 $\mu\ell$ partially purified fraction from *Styela clava* using ultrafiltration (MW CO 5KDa) (SCU5) treated with 1 ml DMEM containing 1% antibiotic / antimycotic. Vessel rings were cultured for 1 h in a cell culture incubator maintained a 37°C and 5% CO_2. NO metabolites (NOx; nitrite and nitrate) in media were measured by reduction of nitrate by vanadium (III) and detection with Griess reagents (41).

After loading with diluted serum samples (100 ㎕) in 96-well microtiter plate, addition of VCl_3 (100 ㎕) to each well was rapidly followed by addition of the Griess reagents, 2% sulfanilamide (SULF; 50 ㎕) and 0.1% N-(1-naphthyl) ethlenediamine (NEDD; 50 ㎕). After incubation at 37°C, and then read absorbance at 570 nm. And the results were expressed as μM of NOx.

Cell culture

Human endothelial cell line EA.hy926 cells were used transformed human endothelial cell line EA.hy926 cell was kindly provided by Dr. C. Edgell (University of North Carolina at Chapel Hill, NC). Cells were cultured at 37 °C in Dulbecco modified eagel medium (Invitrogen, Carlsbad, CA, USA) supplement with 10% fetal bovine serum (FBS) and antibiotics (penicillin G and stereptomycin).

Measurements of nitrie and nitrate (NOx) in cell cultured media

NO metabolites (NOx; nitrite and nitrate) in cell cultured media were measured by reduction of nitrate by vanadium (III) and detection with Griess reagents (41). After loading in 96-well microtiter plate with diluted serum samples (100 ㎕), addition of VCl_3 (100 ㎕) to each well was rapidly followed by addition of the Griess reagents, 2% sulfanilamide (SULF; 50 ㎕) and 0.1% N-(1-naphthyl) ethlenediamine (NEDD; 50 ㎕). After incubation at 37 °C, and then read absorbance at 570 nm. And the results were expressed as μM of NOx.

Immunofluroescence Analysis

EA.hy926 cells were seeded on chamber slide (Lab-Tek) and grown to 80-90% confluence. Cells were fixed using cold acetone and permeabilized with 0.01% Triton X-100. After blocking the nonspecific site with 5% Bovine serum albumin, the chamber was then incubated with specific rabbit polyclonal anti-phospho-eNOS (1:100, Cell signaling, USA) at 2 hour. Secondary antibody was Alexa 488 (Molecular probes, goat anti-rabbit conjugated IgG 1 : 400, Invitrogen, USA) for 30min. Nuclei were visualized using 1.5 uM propidium iodide (SIGMA, USA).

Statistics

Results were expressed as means ± S.E.M. The statistical significance of difference between the groups were determined using one-way analysis of variance (ANOVA) followed by the Duncan test. P values lower than 0.05 were considered signification.

RESULTS

ACE inhibitory activity

Partial purification was performed using enzyme hydrolysis of *Styela clava*, ultrafiltration (MW CO 5KDa), and molecular exclusion chromatography (Sephadex G-25). And the first fraction of molecular exclusion chromatograph (SCF1) was used for the experiments.

To observe the effect of partially purified fraction from *Styela clava* (SCF1) on angiotensin II converting enzyme (ACE), which induces endothelial dysfunction, ACE inhibitory activity of SCF1 was measured. ACE inhibitory activity of SCF1 was increased in its concentration-dependent manner (0.187 to 3 mg/ml) with the maximum inhibition of 97.54 ± 0.45% at concentration of 3 mg/ml (Figure 18).

SCF1 exhibited higher ACE inhibitory activity than crude extract of *Styela clava* (data not shown). The result indicates that the partially purified fraction from *Styela clava* improves ACE inhibitory activity.

Effects of SCF1 on Vasorelaxations

Effects of partially purified fraction from *Styela clava* (SCF1) on vasorelaxation was observed using rat thoracic aorta prepared with or without endothelium to see the improvement of endothelium-dependent vasorelaxation by SCF1. Vasorelaxation of SCF1 was increased in its concentration-dependent manner (0.187 to 3 mg/ml) with the maximum relaxation of 82.98 ± 6.6% at concentration of 3 mg/ml in aortic rings prepared with endothelium (Figure 19). Without endothelium, however, the vasorelaxation of SCF1 was

reduced at concentration of 3 mg/ml in aortic rings (data not shown). Nitric oxide synthase inhibitor, $_{L}$-NAME (100 uM), attenuated the SCF1 induced relaxation to 36.95 ± 1.4% in the endothelium-intact aorta, suggesting the role of NO in the endothelial-dependent vasorelaxation (Figure 19). The results suggest that more than 45% of vasorelaxtion by SCF1 is NO dependent in aorta.

Effects of SCF1 on blood pressure

Basal arterial blood pressure was observed in SD rats after injection of SCF1. Injection of SCF1 (50 mg/kg) significantly reduced direct arterial blood pressure (Figure 20). SCF1 reduced blood pressure more than 10% of the maximum blood pressure at a concentration of 50 mg/kg. Overall results suggest that SCF1 decreases the blood pressure *in vivo* system.

Effects of SCF1 on NO release from artery

To observe the effects of *Styela clava* on NO production, rat thoracic aorta was prepared with endothelium, was incubated in media, and the levels of NO released were measured at varying concentration of partially purified fraction from *Styela clava* using ultrafiltration (MW CO 5KDa) (SCU5). As shown in Figure 21, the levels of NO released in the incubation media of aortic rings were increased by SCU5 treatment in its concentration dependent manner (0.75 – 3 mg/ml). Without endothelium, however, NO production by SCU5 was not observed in aorta prepared without endothelium (data not shown). The result suggests that *Styela clava* directly affects NO production in endothelium of aorta.

Effects of SCF1 on NO production from EA.hy926 cells

To observe the effects of *Styela clava* on NO production, endothelial cell (EA.hy 926cells) was incubated in medium and the levels of NO released were measured at varying concentration of partially purified fraction from *Styela clava* (SCF1). As shown in Figure 22, the levels of NO released in the incubation media of endothelial cell were increased by SCF1 treatment in its concentration dependent manner (0.75 – 3 mg/ml). The result suggests that *Styela clava* directly affects NO production in endothelial cell.

Effects of SCF1 on eNOS phosphorylation in EA.hy926 cells

Since SCF1 enhanced NO production in aorta, the present study was further investigated the effect of SCF1 on NO synthase activation. Based on immunofluorescence analysis, SCF1 markedly increased eNOS phosphorylation (Ser 1177) level in endothelial cells whereas no significant change of eNOS phosphorylation was observed in cells treated with PBS (Figure 23). Overall data indicates that enhanced NO production in endothelial cell results from increased eNOS phosphorlyation by SCF1.

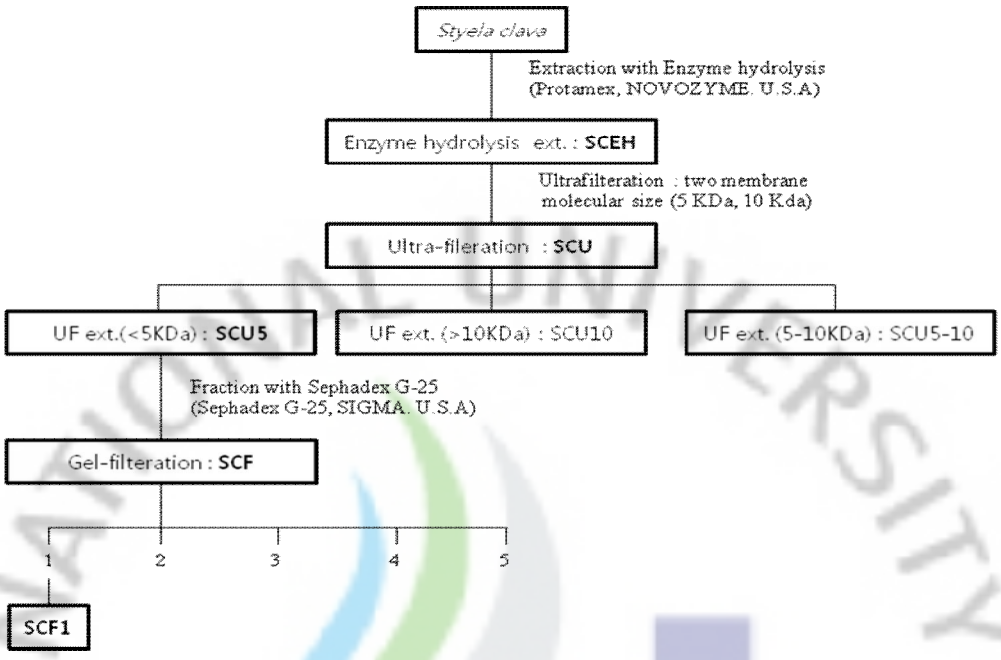


Figure 17. Schematic representation of extraction and partial purification steps of *Styela clava*.

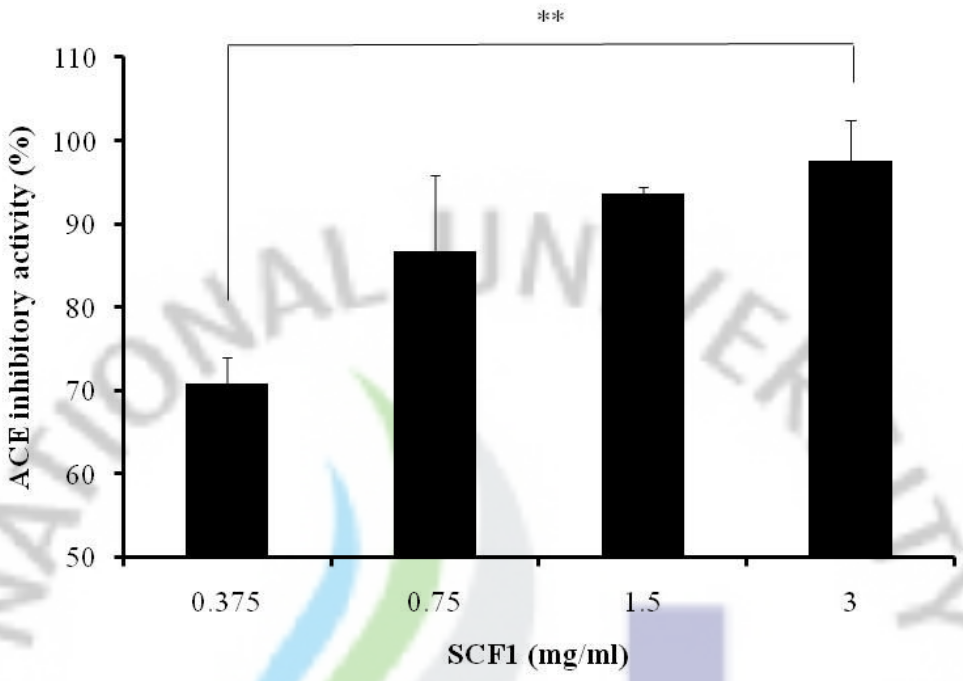


Figure 18. Angiotensin - II converting enzyme (ACE) inhibitory activity of partially purified fraction from *Styela clava* (SCF1). protein concentration was ranged from 0.187 mg/ml to 3 mg/ml. Data are means ± S.E.M. **p < 0.01 compared with low dose concentration of SCF1.

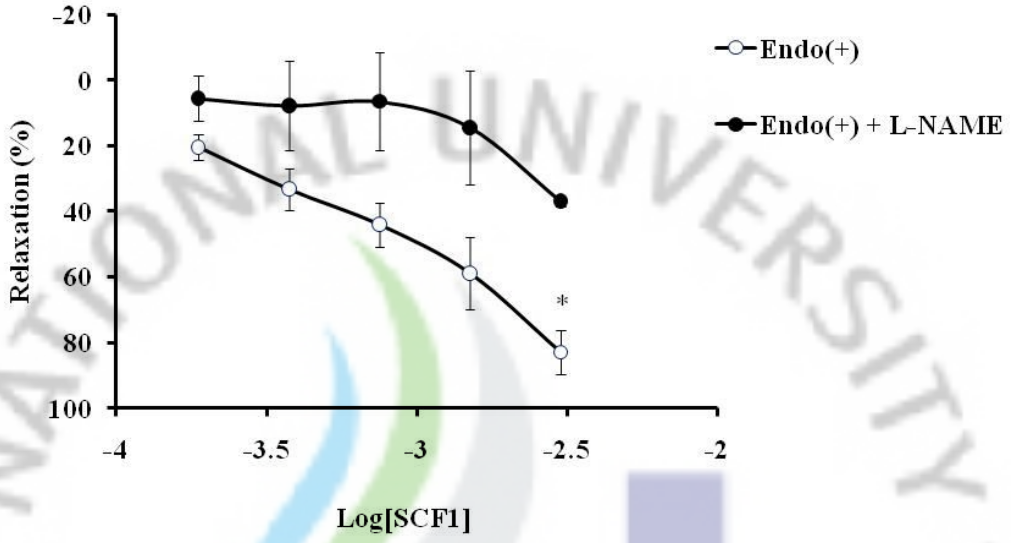


Figure 19. Endothelial-dependent vasorelaxation by SCF1. Rat thoracic aortic rings with endothelium, and with endothelium plus $_{L}$-NAME (100 uM) preincubation were prepared, and vasorelaxation effects of SCF1 (from 0.187 to 3.0 mg/ml) were observed. Data are means ± S.E.M. *p < 0.05 and **p < 0.01 compared with GBcon.

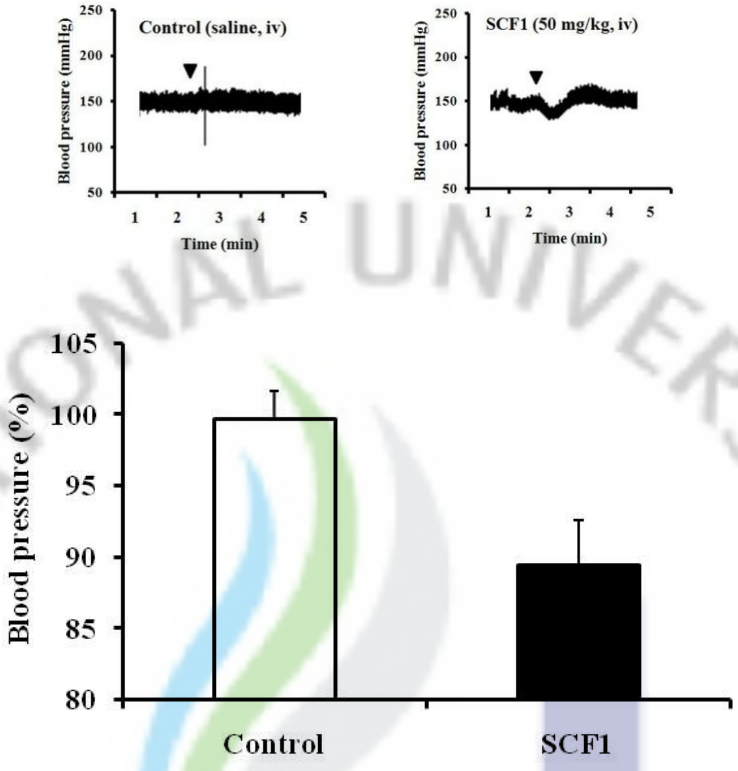


Figure 20. Effects of SCF1 on direct blood pressure in SD rat. Basal arterial blood pressure (MAP) was observed in SD rats as described in Materials and Methods.

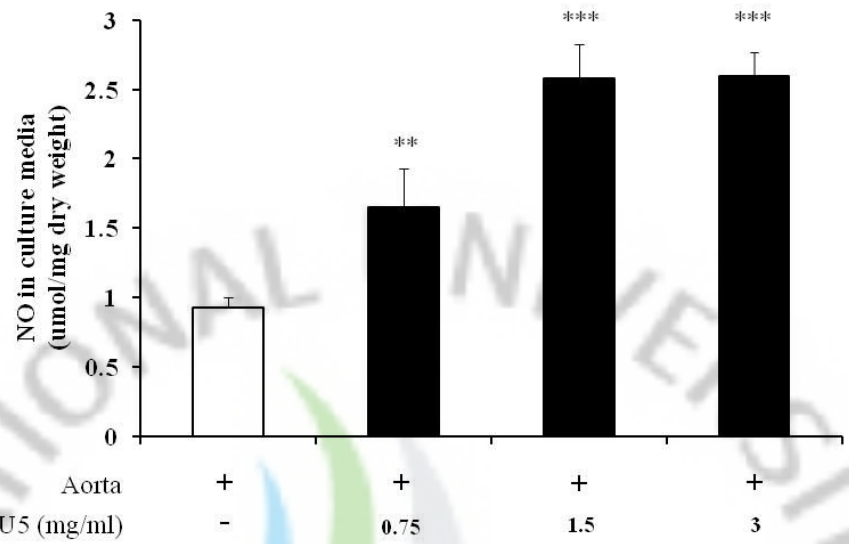


Figure 21. Effects of SCU5 on nitric oxide (NO) released from isolated thoracic artery. The NO concentration was determined by detection of total nitrite and nitrate released from isolated thoracic artery treated with SCU5 in media. Data are means ± S.E.M. **p < 0.01 and ***p < 0.001 compared with non treated group

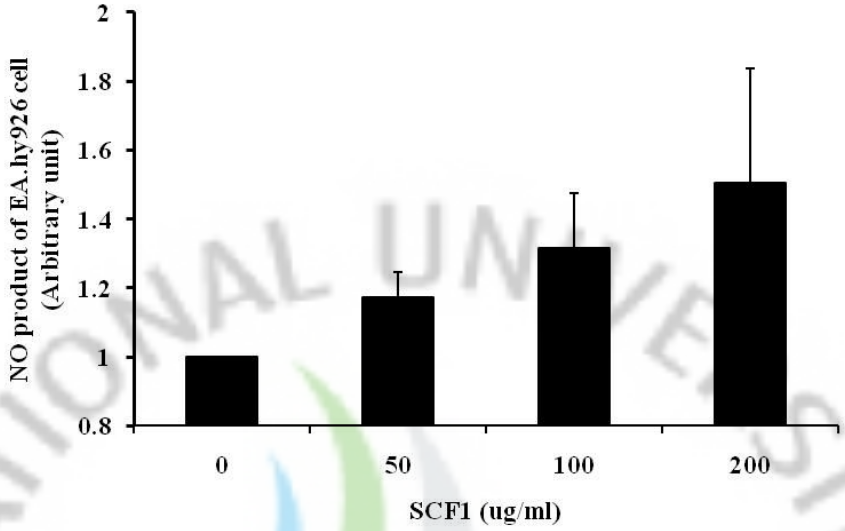


Figure 22. Effects of SCF1 on nitric oxide (NO) production from EA.hy926 cells. The NO concentration was determined by detection of total nitrite and nitrate released from EA.hy926 cells treated with SCF1 in media.

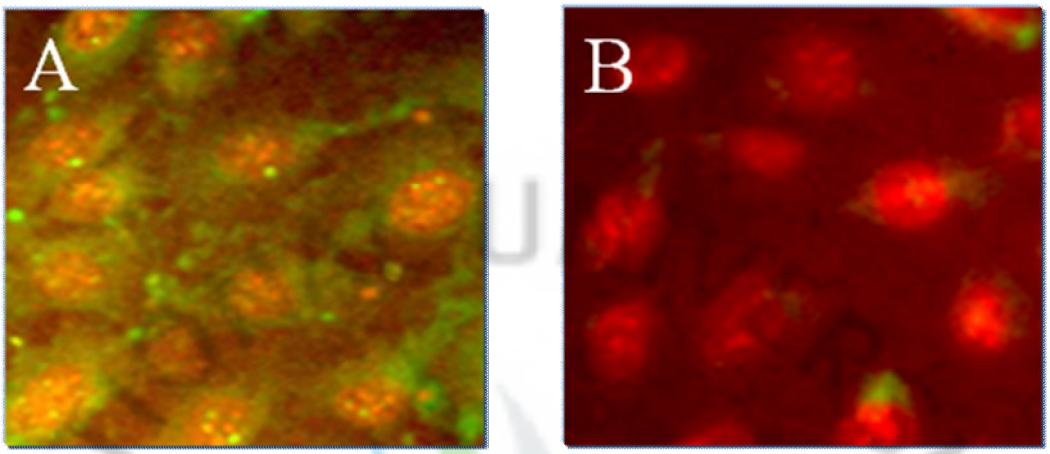


Figure 23. Effects of SCF1 on eNOS Phosphorylation (Ser 1177) in EA.hy926 cell. EA.hy926 cells were treated with SCF1 (A) and PBS (B) for 24 hrs, and were fixed with acetone. Anti-phosphos eNOS (Ser 1177) labled with fluorescence is shown in green, and DNA stained with PI is shown in red.

DISCUSSION

In the present study, partially purified fraction from *Styela clava* (SCF1) showed higher inhibitory effect on angiotensin II-converting enzyme (ACE) activity than crude extract from *Styela clava*. SCF1 showed the increased endothelial-dependent vasorelaxation. In addition, the vasrelaxation effect of SCF1 was significantly attenuated by NO synthase (NOS) inhibitor, $_{L}$-NAME, in aorta prepared with endothelium. Treatment of aorta with SCF1 increased the release of NO from endothelial cell, which result from the enhancement of vascular eNOS phosphorylation. Overall results suggest that SCF1 would allow the development of new therapeutic drug for hypertension based on endothelial-dependent vasorelaxation mechanism.

In the present study, purified fraction from *Styela clava* (SCF1) showed higher inhibitory effect on angiotensin II-converting enzyme (ACE) activity than crude extract of *Styela clava*. The previously studies have suggested that endothelial dysfunction may be therapeutically reversible (8), and ACE inhibitors and angiotensin receptor antagonist were found to improve flow-evoked endothelium-dependent vasorelaxation (9, 21, 47, 55). Angiotensin II has been implicated in the pathophysiology of vascular disease. Production of Ang II begins with conversion of angiotensinogen to angiotensin I by rennin, which is transformed to Angiontensin II by the catalyzing action of the ACE (50). Ang II decreases NO level by promoting oxidative stress (15, 50), increases ROS by stimulating NADPH oxidase (62), and promotes vascular inflammation (57). Angiotensin II can stimulate the vascular endothelial cells to increase the production of active oxygen, and increase the oxidative stress level in plasma (62). In hypertensive patients, interruption of the rennin-angiotensin system with ACEI of Angiotensin II receptor blockers restores endothelial function (56, 57). In this study, SCF1 showed the ACE inhibitory effects which might be

relevant to increased NO production and decrease superoxide generation by SCF1

In the present study, the effects of partially purified fraction from *Styela clava* (SCF1) on vasorelaxation were observed using rat thoracic aorta prepared with or without endothelium. The endothelial dependent vasorelaxation activity of SCF1 was reduced by removal of endothelial cells in aorta. Moreover, blockade of the NO synthase with $_{L}$-NAME casused a significant inhibition of the relaxant effect of SCF1.

Vasorelaxation mechanisms may be characterized as either endothelium-dependent or -independent (30). Acetylcholine and shear stress are usually used for inducing endothelium-dependent vasorelaxation, whereas sodium nitroprusside (SNP) is used for inducing endothelium-independent vasorelaxation (EIDV) (45).

Nitric oxide is the endothelium-derived relaxing factor. Furchgott and Zawadzki (1980) showed that the normal relaxation response to acetylcholine was abolished when the endothelial lining was rubbed off (20). Palmer et al. (1987) identified the endothelium-derived relaxation factor is NO, which is known to be the primary endogenous vasodilator (45). $_{L}$-NAME is a prodrug which has NO synthase inhibitory activity and is rapidly hydrolyzed in biological tissues (61). Nitric oxide is generated from L-arginine by the calcium/calmodulin-dependent enzyme, nitric oxide synthase (NOS), in the site of vascular endothelium (39). After synthesis, NO diffuses out of the endothelial cells, and traverses membranes of vascular smooth muscle cells (VSMC) (45). Nitric oxide stimulates guanylate cyclase activity in target cells, and increases cGMP level which results in muscle relaxation via decreased intracellular calcium (Ca^{2+}) (22). In this study, SCF1 induced the endothelial-dependent vasorelaxation in aorta by increasing NO production which might result from enhanced eNOS phosphorylation (Ser1177) by SCF1.

Effects of SCF1 on Nitric oxide production observed using human endothelial cell (EA.hy926cell). In endothelial cells (EA.hy926 cell), NO synthesis was increase and eNOS phosphorylation (Ser 1177) was increased by partially purified fraction from *Styela clava*. In

addition, Immunocytochemical data demonstrated that active compound isolated from *Styela clava* enhanced eNOS phosphorylation in endothelial cell cytosol than control. Which suggest that increased NO production results from enhanced expression of eNOS phosphorylation in endothelial cell. Injuries of dysfunctional endothelial cells with a loss of endothelium-derived nitric oxide (NO) are major critical factors in the pathogenesis of vascular disease, including hypertension. Also, the main source of endothelial NO, a crucial factor for the normal functioning of the cardiovascular system, is eNOS expressed by endothelial cells (26). eNOS is mainly located in the vascular endothelium. Moreover, eNOS has been traditionally characterized as a calcium/calmodulin-dependent constitutive enzyme, in the site of vascular endothelium (14). Endothelial NO is produced by eNOS, which is classically activated by agonists such as acetylcholine, histamine and bradykinin inducing an increase in intracellular calcium levels followed endothelial dependent modulation (68). After synthesis by phosphorylated eNOS, NO diffuses out of the endothelial cells, and traverses membranes of vascular smooth muscle cells (VSMC) (45). Nitric oxide stimulates guanylate cyclase activity in the target cell, and increases cGMP level which results in relaxation of smooth muscle via decreased intracellular calcium (Ca^{2+}) (22). Moreover, cGMP activates cGK-1, which mediates vascular relaxation via phosphorylation of several proteins regulating intracellular $Ca2^{+}$ - mobilization (49). Previous studies demonstrated that the pharmacological actions of the Ca^{2+} channel antagonists, Amlodipine, in eNOS/NO cascade system (54, 37). Triggering of eNOS/NO cascade system leads to vasorelaxation by decreasing intracellular concentration of Ca^{2+} in smooth muscle (37). The present study showed that increased NO production by partially purified fraction of *Styela clava* in aorta and endothelial cell. Which suggest that increased NO production from enhanced expression of eNOS phosphorylation in aorta and endothelial cell. These findings supported that bioavailability of NO is selectively decreased blood pressure

In this study, partially purified fraction from *Styela clava* (SCF1) induced the

endothelial-dependent vasorelaxation in aorta by increasing NO production which might result from enhanced eNOS phosphorylation (Ser1177). In addition, the ACE inhibitory effect of SCF1 might increase NO production and decrease superoxide generation. The effect of SCF1 on the endothelial dependent vasorelaxation might result from increased NO bioavailability by enhanced eNOS/NO cascade system. Further study should be investigated to evaluate active compound in *Styela clava*. Overall results suggest that the partially purified fraction from *Styela clava* (SCF1) can be applied for the development of new therapeutic drugs.

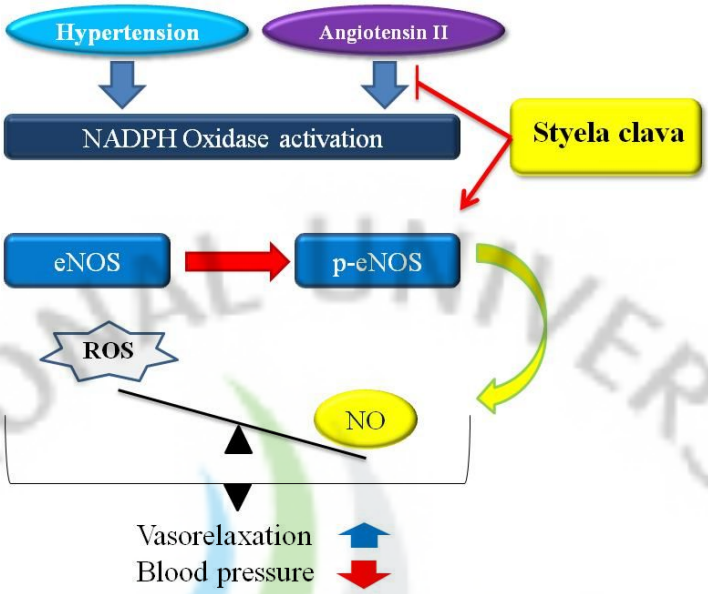


Figure 24. Proposed mechanism of endothelial-dependent vaorelaxation by partially purified fraction from *Styela clava*.

CONCLUSION

In this study, GB166 and SCF1 induced the endothelial-dependent vasorelaxation in aorta by increasing NO production which might result from enhanced eNOS phosphorylation (Ser1177). In addition, the ACE inhibitory effect of GB166 and SCF1 might increase NO production and decrease superoxide generation. The effect of GB166 on the endothelial dependent vasorelaxation might result from increased NO bioavailability by enhanced eNOS/NO cascade system, and decreased O_2^- generation by reduced expression of NADPH oxidase subunit, $p47^{phox}$, in aorta. The structural changes (e.g. hydroxylation or carboxylation) of metabolites in GB166 might cause the increased pharmacological activity of original Gwibi-tang. Also, the effect of SCF1 on the endothelial dependent vasorelaxation might result from increased NO bioavailability by enhanced eNOS/NO cascade system. Overall results suggest that the biotransformation of herbal medicine and SCF1 can be applied for the development of new therapeutic drugs.

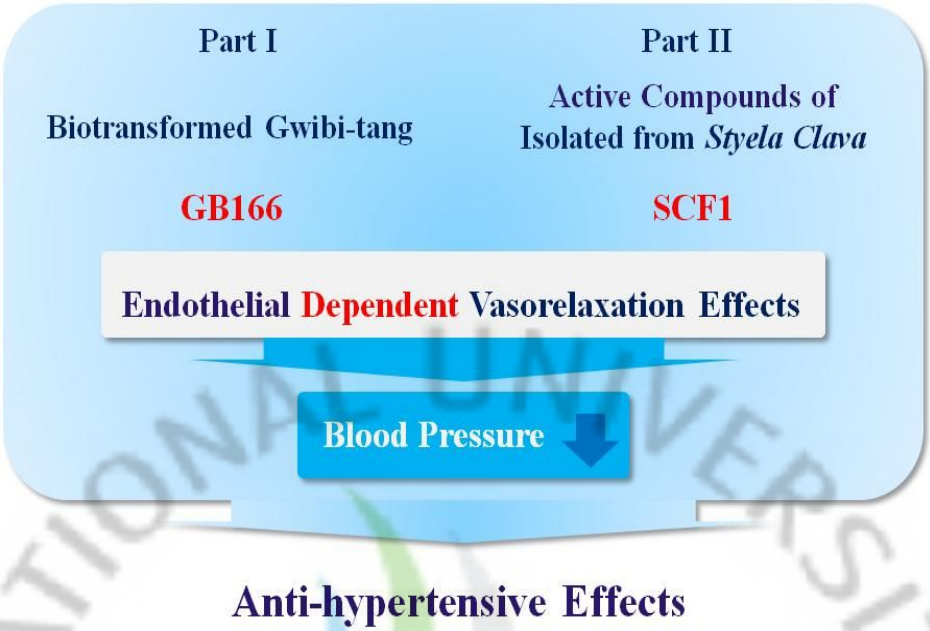


Figure 25. Summary of antihypertensive effects of GB166 and partially purified fraction from *Styela clava* (SCF1).

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