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Vasorelaxing Activity of *Ulmus davidiana* Ethanol Extracts in Rats: Activation of Endothelial Nitric Oxide Synthase

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***Ulmus davidiana* var. *japonica* Rehder (Urticales: Ulmaceae) (UD) is a tree widespread in northeast Asia. It is traditionally used for anticancer and anti-inflammatory therapy. The present study investigated the effect of an ethanol extract of UD on vascular tension and its underlying mechanism in rats. The dried root bark of UD was ground and extracted with 80% ethanol. The prepared UD extract was used in further analysis. The effect of UD on the cell viability, vasoreactivity and hemodynamics were investigated using propidium iodide staining in cultured cells, isometric tension recording and blood pressure analysis, respectively. Low dose of UD (10～100 μg/ml) did not affect endothelial cell viability, but high dose of UD reduced cell viability. UD induced vasorelaxation in the range of 0.1～10 μg/ml with an ED_{50} value of 2 μg/ml. UD-induced vasorelaxation was completely abolished by removal of the endothelium or by pre-treatment with L-NAME, an inhibitor of nitric oxide synthase. UD inhibited calcium influx induced by phenylephrine and high K^+ and also completely abolished the effect of L-NAME. Intravenous injection of UD extracts (10～100 mg/kg) decreased arterial and ventricular pressure in a dose-dependent manner. Moreover, UD extracts reduced the ventricular contractility (+dP/dt) in anesthetized rats. However, UD-induced hypotensive actions were minimized in L-NAME-treated rats. Taken together, out results showed that UD induced vasorelaxation and has antihypertensive properties, which may be due the activation of nitric oxide synthase in endothelium.**

Key Words: *Ulmus davidiana* var. *japonica*, Endothelial nitric oxide synthase, Vasorelaxation, Blood pressure, Ventricular contractility

INTRODUCTION

Vascular endothelial dysfunction reflected by reduced nitric oxide availability is certainly the causative factor or promoting mechanism of arteriosclerosis and hypertension. Nitric oxide produced in endothelium has been implicated in the regulation of blood pressure. As the major regulator of vascular homeostasis, the endothelium exerts a number of vasoprotective effects, such as vasodilation, suppression of smooth muscle cell growth, and inhibition of inflammatory responses [1]. Abnormal endothelial nitric oxide production is closely related with the pathogenesis of atherosclerosis and hypertension [2]. NO derived from L-arginine can be synthesized by activating nitric oxide synthase (NOS). Especially, endothelial nitric oxide synthase (eNOS) is a target protein for treating hypertension in alternative medicine [3].

Ulmus davidiana var. *japonica* Rehder (Urticales: Ulmaceae) (UD) is widely used as traditional medicine for anticancer and anti-inflammatory therapy in Asia [4-6]. Several compounds have been identified in the stem and root barks of UD, and their pharmacological actions have been uncovered [5,7,8]. Recently it was reported that bakuchiol isolated from UD extracts inhibited lipopolysaccharide-induced nitric oxide and prostaglandin in macrophage, suggesting anti-inflammatory action [9]. Moreover, UD inhibited ovalbumin-induced oxidative stress and inflammation in a murine asthma model via hemooxygenase-1 upregulation [10] and methanol extracts of UD possessed anti-angiogenic activity [11].

However, the effect of UD on vascular tone which regu-

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ABBREVIATIONS: UD, *Ulmus davidiana* var. *japonica*; eNOS, endothelial nitric oxide synthase; L-NAME, N^G-nitro-L-arginine methyl ester; PE, phenylephrine; ACh, acetylcholine.

lates peripheral resistance of blood pressure control is unknown. The purpose of this study was to investigate the vascular relaxing activity and antihypertensive action of an ethanol extract of UD.

METHODS

Plant material and preparation of ethanol extracts

The root bark of UD was collected from Chungnam Province, Republic of Korea in July 2009 and identified by Prof. Jun Pok Chang (Joongbu University, Department of Herbal Pharmaceutical Science, Korea). A voucher specimen (C5-334-1) was deposited in the herbarium, Joongbu University, Korea. The root bark was washed three times with tap water and stored at −20°C after lyophilization. Dried samples (50 g) were crushed in a blender and the crude powder was extracted with 80% ethanol (EtOH) at 70°C for 3 h under reflux. The EtOH extract was not sequentially fractionated using other organic solvents. For conservation without microbial contamination, the EtOH extract (4.2 g) was passed through 0.2 μm filter paper and evaporated on a rotary evaporator under reduced pressure. The extract was again dried using a freeze-dryer to produce a solid powder (final yield, 12.1%) [12].

Cell cultures and reagent

Mouse MS-1 endothelial cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in Dulbecco-modified Eagle medium (DMEM, Gibco, USA) with 10% fetal bovine serum, 10 U/ml penicillin, and 10 μg/ml streptomycin [13]. Ketamine was purchased from Huons (Korea). Phenylephrine, N^G-nitro-L-arginine methyl ester (L-NAME), acetylcholine, xylazine were obtained from Sigma-Aldrich (St. Louis, MO, USA). All chemicals were dissolved in distilled water except UD which was dissolved in DMSO.

Cell viability

Cell viability of the cultured endothelial cells was measured using an automatic cell counter (ADAM-MC, Digital Bio, Seoul, Korea) which analyzed the viable and non-viable cells after adding propidium iodide (PI). PI intercalates into double-stranded nucleic acids and is excluded by viable cells but can penetrate the cell membranes of dying or dead cells. Cell morphologic change for dying cells was assessed by the microscopic findings showing round detached cells.

Measurement of vasoreactivity in rat aortic rings

Male Sprague-Dawley rats weighing 150∼250 g (n=20) were anesthetized with an intraperitoneal injection of 80 mg/kg ketamine and 12 mg/kg xylazine. All animal procedures were in accordance with the Chungnam National University Guide for the Care and Use of Laboratory Animals. The thoracic aorta was immediately isolated and fat layer and adventitia were removed under stereomicroscopy and cut into 2∼3 mm rings. Each ring was connected to an isometric force transducer (MultiMyograph 610 M, Danish Myo Technology, Copenhagen, Denmark), suspended in an organ chamber filled with 7.5 ml of Kreb's buffer solution (NaCl 100 mM, KCl 4.7 mM, $CaCl_2$ 1.9 mM, $MgSO_4$ 1.2 mM, K_2HPO_4 1.03 mM, $NaHCO_3$ 25 mM, Glucose 11.1 mM, pH 7.4), and aerated with 95% O_2/5% CO_2 [13,14]. Isometric tension was recorded continuously. After 1 h equilibration period, the rings were precontracted with phenylephrine (PE, 0.3 μM) or high K^+ (60 mM) and then endothelium-dependent relaxation was observed with the cumulative addition of extract (1 μg/ml∼1 mg/ml). Relaxation was expressed as a percentage of the precontracted tension obtained with PE. In some cases, the vasorelaxation was tested in aortic rings in which the endothelium was removed. The presence or lack of functional endothelium was examined by demonstrating the presence or absence of relaxation induced by acetylcholine (ACh, 1 μM) in vascular rings precontracted with PE (0.3 μM).

Hemodynamic measurements

Rats weighing 200∼250 g (n=15) were anesthetized with an intraperitoneal injection of urethane (1 mg/g). A Millar catheter (SPR-320, Millar Instruments, Houston, TX, USA) was inserted into the right carotid artery and advanced into the left ventricle. Polyethylene tubing (PE-50) was inserted into the left femoral artery for measuring arterial pressure and into the right jugular vein for fluid or drug administration [15]. After a 20 min stabilization period, signals were continuously recorded at a 1 kHz sampling rate by Powerlab (Adinstruments, Mountain View, CA, USA). Hemodyanamic parameters such as blood pressure, heart rate, and the contractility index were analyzed with Chartpro software (Adinstruments, Mountain View, CA, USA).

Statistical analysis

The data are expressed as mean±S.E. Statistical sig-

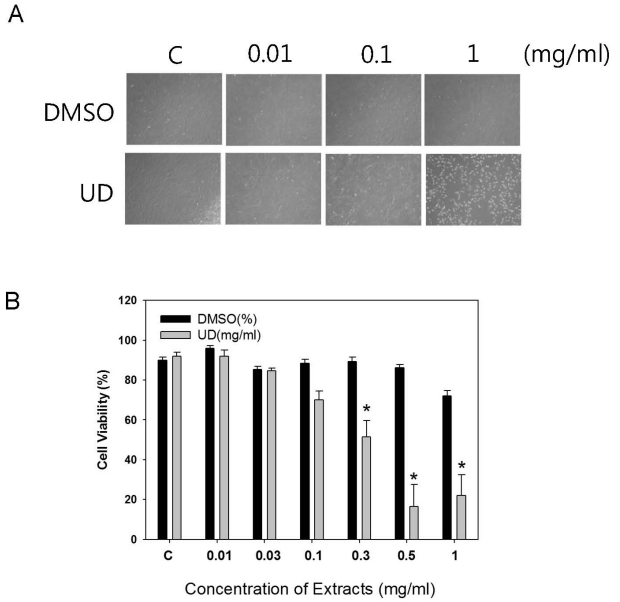

Fig. 1. Effect of an ethanol extract of *Ulmus davidiana* var. *japonica* (UD) on endothelial cell viability. (A) Representative endothelial cell morphology 24 h after exposure to UD (0.01∼1 mg/ml). (B) Cell viability was assessed with propidium iodide staining. Each bar shows the mean±S.E. (n=4). C: untreated control, *$p<0.05$ versus control.

nificance was analyzed by one-way analysis of variances followed by a Turkey post hoc test using SPSS version 17.0 and p-values < 0.05 were considered significant.

RESULTS

Effect of UD on endothelial cell viability

The effect of UD on cell viability was assessed in MS-1 endothelial cells after 24 h incubation. After the endothelial cells were exposed to various concentrations of UD extract (0.01～1 mg/ml) for 24 h, cell viability was measured by PI staining. As shown in Fig. 1A, DMSO as a UD vehicle did not affect endothelial cell morphology; however, 1 mg/ml UD caused marked changes in endothelial morphology, suggesting a decrease in cell viability. PI staining showed that UD (0.1～1 mg/ml) significantly decreased cell viability in a dose-dependent manner, compared with vehicle (Fig. 1B). Thus, we used 10 μg/ml or less UD in the vascular tension experiments.

Effect of UD on vasocontraction in the rat aorta

UD significantly relaxed the aortic rings precontracted with PE (0.3 μM) in a concentration-dependent manner (0.1 ～10 μg/ml). Concentrations greater than 3 μg/ml of UD resulted in maximal relaxation and resulted in an ED_{50} value of 2 μg/ml. UD also relaxed the aortic rings preconstricted with high K^+ (60 mM). However, the vasorelaxing effect of UD was greater in the PE contracted rings, compared with the depolarizing solution contracted rings. UD-induced relaxation was completely abolished by removing the endothelium (Fig. 2A), indicating that UD-induced relaxation is entirely endothelium-dependent.

Next, we investigated the effect of L-nitroarginie methyl ester (L-NAME), an endothelial nitric oxide synthase inhibitor, on UD-induced vasorelaxation. In endothelium-intact aortic rings, pretreatment with 0.1 mM L-NAME completely inhibited the relaxation induced by the UD extracts preconstricted with PE or high K^+ solution, suggesting that UD-induced relaxation is mediated by the activation of ni-

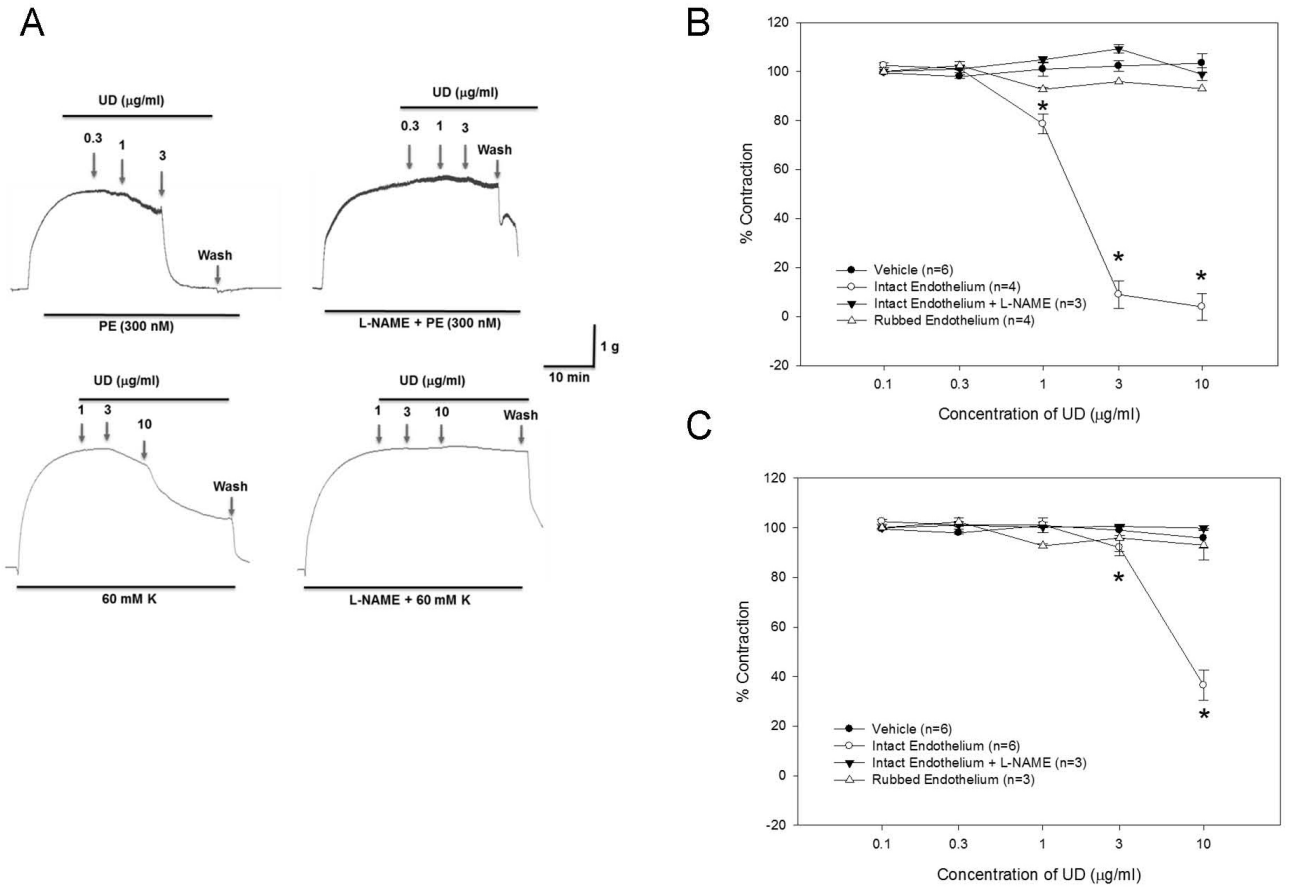

Fig. 2. Vasorelaxing effects of an ethanol extract from *Ulmus davidiana* var. *japonica* (UD) in rat aortic rings. (A) Typical tracing of the vasorelaxing effect of UD on endothelium-intact rings. UD evoked vasorelaxation in phenylephrine (300 nM) or high K^+ (60 mM)-preconstricted aortic rings. Pretreatment with 0.1 mM L-nitroarginine methyl ester (L-NAME) abrogated UD-induced vasorelaxation. (B) Summarized data for the vasorelaxing effect of UD on phenylephrine-induced contraction. (C) Summarized data for the vasorelaxing effect of UD on high K^+ (60 mM)-induced contraction. Vehicle: In endothelium intact rings with DMSO as a vehicle. Intact Endothelium: In endothelium intact rings. Intact endotheium+L-NAME: In endothelium-intact rings pretreated with L-NAME. Rubbed endothelium: In endothelium rubbed rings. Contraction is represented as % contraction of each maximal contraction. Data are the mean±S.E. *$p < 0.05$ versus vehicle.

tric oxide synthase in the endothelium (Fig. 2).

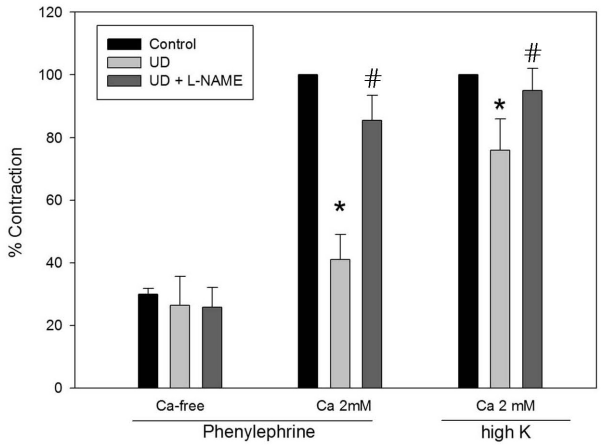

Fig. 3. Effect of an ethanol extract of *Ulmus davidiana* var. *japonica* (UD) on intracellular Ca^{2+} release and Ca^{2+} influx in rat aortic rings. Phenylephrine-induced contractions were evoked in the absence or presence of extracellular Ca^{2+} and high K^+-induced contractions were evoked by the addition of extracellular Ca^{2+}. Note: High K^+-induced contractions were not evoked in the absence of extracellular Ca^{2+}. The aortic rings were pretreated with UD (10 μg/ml) or L-nitroarginine methyl ester (L-NAME) for 10 min before the exposure to phenylephrine or high K^+. Each bar shows the mean ±S.E. (n=5) *$p<0.05$ versus control. #$p<0.05$ versus UD alone.

The effect of UD on Ca^{2+} entry: Involvement of NO

To verify whether UD-induced relaxation of the aorta involve a Ca^{2+} entry blockade, PE-induced contraction in Ca^{2+}-free Kreb's solution and the effects of Ca^{2+} addition were compared in the absence or presence of UD extracts in the aortic rings with intact endothelium. UD did not affect the PE-induced intracellular Ca^{2+} release as assessed PE-induced contraction in Ca^{2+}-free Kreb's solution but markedly suppressed Ca^{2+}-induced contraction in the rat aorta incubated in the PE-containing Ca^{2+}-free Kreb's solution. Also, pretreatment of UD (10 μg/ml) inhibited Ca^{2+}-induced contraction in the high K^+ (60 mM)-containing Ca^{2+} free Kreb's solution (Fig. 3). UD-induced suppression on the Ca^{2+}-induced contraction was significantly inhibited by the pretreatment of L-NAME (0.1 mM), suggesting the involvement of endothelial nitric oxide synthase.

Effect of UD extracts on blood pressure in anesthetized rats

The changes in mean blood pressure and heart rate induced by UD extracts were measured in anesthetized normotensive rats. Typical tracing of intravenous injection of UD extracts (100 mg/kg) is shown in Fig. 4A. UD extracts transiently decreased arterial blood pressure, and then low-level arterial pressure was returned to the initial level for 1 h. UD (10～100 mg/kg, i.v) significantly decreased mean blood pressure and ventricular pressure in a dose-dependent manner (Fig. 4B, C). The change in heart rate fol-

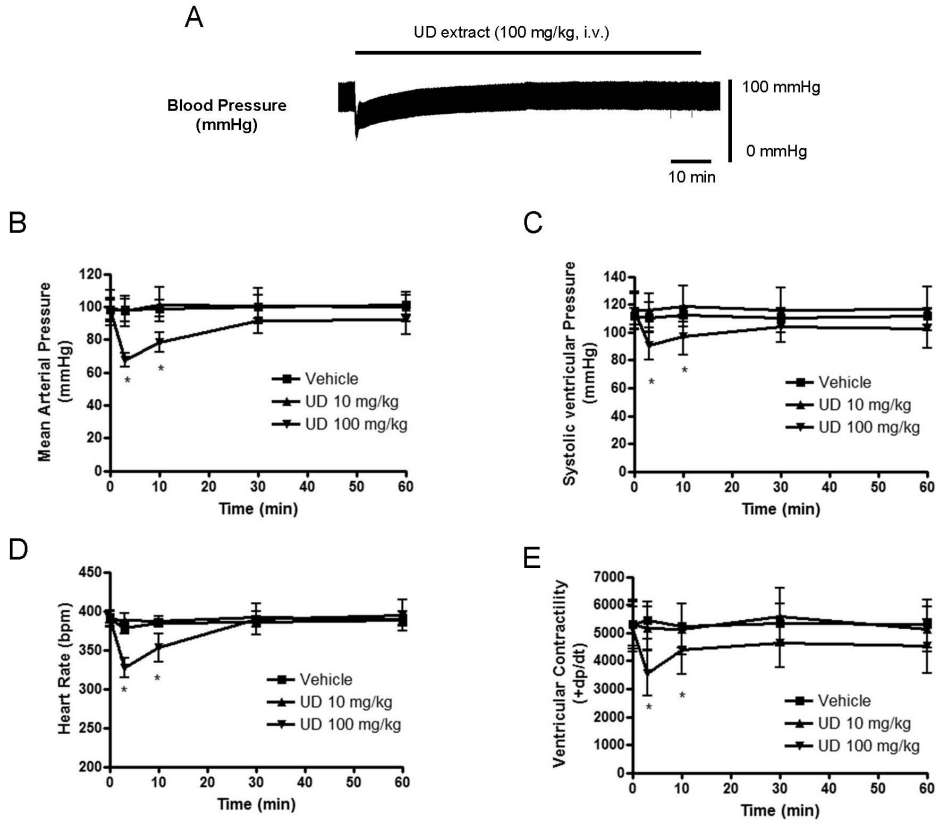

Fig. 4. Effect of an ethanol extract of *Ulmus davidiana* var. *japonica* (UD) on hemodynamics in anesthetized rats. (A) Typical tracing for the change of arterial blood pressure for 1h after intravenous injection of UD (100 mg/kg). (B) Summarized data of the change of mean arterial pressure (B), ventricular pressure (C), heart rate (D), and ventricular contractility (E) after intravenous injection of UD (10～100 mg/kg). UD was intravenously injected into the jugular vein of rats. Data are the mean±S.E. (n=5～7). *$p<0.05$ versus vehicle.

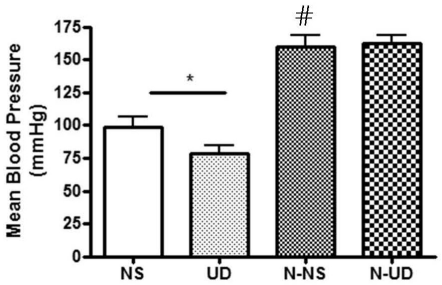

Fig. 5. Effect of an ethanol extract of *Ulmus davidiana* var. *japonica* (UD) on mean arterial blood pressure in anesthetized rats. Mean arterial blood pressure which was taken in 10 min after injection of normal saline (NS), 100 mg/kg of UD (UD), L-NAME+ normal saline (N-NS) and L-NAME+100 mg/kg of UD (N-UD) in anesthetized rats. UD or normal saline were intravenously injected into the jugular vein of rats. L-NAME (40 mg/kg) was injected in intraperitoneum. Data are the mean±S.E. (n=4~5). *$p<0.05$ for NS versus UD. #$p<0.05$ for NS versus N-NS.

lowing the administration of the UD extracts reached a maximum 3 min after intravenous injection and slowly recovered to the initial level. Moreover, after the intravenous injection of UD, +dP/dt, as an index of myocardial contractility reduced, suggesting suppression of left ventricular function as shown by a decrease in +dP/dt (Fig. 4E).

Effect of L-NAME on UD-induced hypotensive action

To know the possible role of nitric oxide on UD-induced hypotensive action, we investigated the hypotensive effect of UD in the L-NAME-treated rats. Administration of L-NAME (40 mg/kg, i.p.) increased the mean arterial blood pressure in the anesthetized rats (96±7 mmHg for normal rats versus 160±9 mmHg for L-NAME-treated rats). As shown in Fig. 5, UD-induced hypotensive effects, which was measured in 10 min after injection of UD or vehicle, were abrogated in the L-NAME-treated rats compared with normal rats. It is suggesting that nitric oxide which is evoked by UD would be main mediator on UD-induced hypotensive action in rats.

DISCUSSION

The present study clearly demonstrated that UD ethanol extracts induced vasorelaxation in an endothelium- and eNOS-dependent manner. Removal of the endothelium abolished the relaxant response to UD, indicating that vasorelaxation by UD was endothelium-dependent. Furthermore, pretreatment with the nitric oxide synthase inhibitor, L-NAME, completely blocked UD-induced vasorelaxation and hypotensive action. These findings suggest that UD-induced vasorelaxation is closely associated with the activation of endothelial nitric oxide synthase.

The mechanism for the increase in cytosolic Ca^{2+} levels in vascular smooth muscle is explained by two different calcium influx pathways: receptor-linked calcium channels and voltage-dependent calcium channels [16,17]. Interaction of the contractile agonists, such as PE, with their receptors induces the generation of inositol-1,4,5-triphosphate (IP_3) and diacylglycerol, which activate protein kinase C [18]. IP_3 subsequently binds to its receptor in the sarcoplasmic reticulum. This releases calcium from the sarcoplasmic reticulum to induce an initial transient contraction and subsequently opens the receptor-linked calcium channels, which may be activated by IP_3 [18]. Ligands such as PE also induced the cation influx, which may lead to membrane depolarization in the endothelial cells [19,20]. In contrast, high K^+ induced membrane depolarization, which opened voltage-dependent calcium channels, resulting in an increase in Ca^{2+} influx [21]. Our data showed that UD inhibited vasocontraction via an inhibition of extracellular Ca^{2+} influx, not intracellular Ca^{2+} release. Also UD-induced inhibition of Ca^{2+} influx was inhibited by L-NAME, indicating that it was mediated by NO. These results suggest that UD inhibits Ca^{2+} influx through calcium channels and not affect release of Ca^{2+} from intracellular stores. It is well known that nitric oxide decreases intracellular Ca^{2+} in vascular smooth muscle by inhibition of Ca^{2+} entry and did not affect release of Ca^{2+} from intracellular stores [22]. Therefore, our findings suggest that UD-induced vasorelaxation is closely associated with the activation of endothelial nitric oxide synthase.

As we established that UD has a vasorelaxing effect in rat aorta, we next evaluated the hypotensive effect of UD on anesthetized rats. Our data showed that an intravenous injection of UD extract decreased blood pressure and ventricular +dP/dt max in a dose-dependent manner. Several humoral and neural factors are involved in blood pressure control. Our data established that UD induced vasorelaxation which depends on vascular endothelium, therefore, hypotensive effect of UD extracts was likely associated with the activation of vascular endothelium. Another possibility for hypotensive effect of UD extracts is the involvement in heart. NO is produced in at least two major cell types in the heart: endothelial and cardiac myocytes. eNOS gene deletion enhances the inotropic response not only to isoproterenol stimulation but also under basal conditions. These results suggest that eNOS blunts the inotropic response to isoproterenol stimulation and that its genetic absence enhances contractility [23]. Therefore, our data suggested that a high UD dose reduced ventricular contractility, which may be related to increased nitric oxide production via endothelial nitric oxide synthase activation in vascular endothelium. To know the possible role of nitric oxide on UD-induced hypotensive action, we investigated the hypotensive effect of UD in the L-NAME-treated rats. UD-induced hypotensive effects were abrogated in the L-NAME-treated rats. Therefore our results suggest that nitric oxide which is evoked by UD would be main mediator on UD-induced hypotensive action in rats.

Investigations of the phytochemical components of UD have shown in the isolation of catechin [24], triterpene esters [25], sesquiterpene O-naphthaquinones [26], and lignan and neolignan glycosides [27]. Among the phytochemicals, epigallocatechin-3-gallate activates eNOS by Akt-dependent pathway and might be involved in endothelial-dependent vasorelaxation [28].

In our study, low dose of UD might improve the endothelial function via the activation of endothelial nitric oxide synthase, however, long-term treatment with high dose of UD extracts is not recommended as it may produce a potential risk of endothelial damage. Taken together, out data showed that UD induced endothelium-dependent vasorelaxation and has antihypertensive properties in the rats. Therefore, the vasorelaxing effects of UD may be useful for the treatment of endothelial dysfunction or hypertension.

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