Antihypertensive, Vasorelaxant, and Antioxidant Effect of Root Bark of *Ulmus macrocarpa*

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The present study was performed to evaluate the cardiovascular effects of ethanolic extract from the root bark of *Ulmus macrocarpa* (RBUM) in rats. The effects of RBUM on the vascular response of isolated rat aorta and the blood pressure of spontaneously hypertensive rats (SHRs) were evaluated. In addition, its antioxidant activity in H9c2 cells was investigated. In the free radical scavenging assay using 1,1-diphenyl-2-picrylhydrazyl stable free radical (DPPH), RBUM exhibited significant scavenging activity with an EC$_{50}$ value of 14.3 µg/ml. RBUM also induced resistance to hydrogen peroxide-mediated oxidative insult in H9c2 myocardial cells. In isolated rat aortic preparations, RBUM exhibited potent vascular relaxant effect with an EC$_{50}$ value of 1.9 µg/ml. This relaxation was significantly inhibited by denudation of the endothelial layer, pretreatment with N²-nitro-L-arginine methyl ester (10 µM), raising extracellular K$^+$ (45 mM), and pretreatment with tetraethylammonium (10 mM). In an antihypertensive study with SHRs, long-term administration with RBUM (100 mg/kg) for 42 d decreased systolic blood pressure (approximately 20 mmHg). In SHRs after 42 d of treatment, RBUM recovered aorta relaxation to acetylcholine and sodium nitroprusside, and attenuated lipid peroxidation in liver of SHRs. These results suggest that chronic treatment with RBUM exerts antihypertensive effects in SHRs, and its direct vasorelaxant and antioxidant properties may contribute to reduce elevated blood pressure.

Key words *Ulmus macrocarpa*; antihypertensive; vasorelaxant; antioxidant; spontaneously hypertensive rat

Reactive oxygen species (ROS) play an important pathophysiological role in the development of hypertension. There is some evidence that free radicals such as superoxide anion (O$_2^•$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radical (OH$^–$), which decrease nitric oxide bioavailability, can lead to cardiovascular remodeling. Patients with hypertension demonstrate increased levels of oxidative stress byproducts together with decreased activity of endogenous antioxidant enzymes in blood and mononuclear cells. These patients also have indications of increased oxidative DNA damage when compared with normotensive individuals. Treatment with superoxide dismutase mimetics or antioxidants improves vascular function, regresses vascular remodeling, and reduces blood pressure. Recent studies have shown that a number of plant products including polyphenols, flavonoids, and various plant extracts exert antioxidant effects that might be owing to a combination of vasodilator and antioxidant actions.

The medicinal plant *Ulmus macrocarpa* HANCE (Ulmaceae) is widely distributed in Asia. The stem and root bark of this species has been used as an oriental traditional medicine for the treatment of edema, mastitis, gastric cancer, and inflammation. Previous research has reported that Ulmi cortex (*Ulmus macrocarpa* HANCE) extracts exert anti-anaphylactic action in rats, nitric oxide-producing properties, and inhibitory effect on matrix metalloproteinases and pro-teases of periodontopathogens. Its active ingredient was identified to be procyanidin oligomers, which are a subclass of flavonoids that comprise oligomers of catechin. These procyanidin oligomers occur primarily in several plants such as grape-seed, cocoa, and *Rhamnus lycioides*, and are known to exert many physiological effects including antioxidant, anti-inflammatory, and antihypertensive effects. However, there is little information about the effects of *Ulmus macrocarpa* extracts on pharmacological actions in cardiovascular disease associated with hypertension.

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scavenging activity of RBUM on 1,1-diphenyl-2-picrylhydrazyl stable free radical (DPPH) was assayed according to a modification of the method described previously. One hundred microliters of various concentrations of RBUM (1—100 μg/ml) was mixed with 100 μl of 0.15 mM DPPH in methanol. Absorbance at 520 nm was determined after 30 min. The DPPH radical scavenging activity of the test substance was calculated by the following equation: scavenging activity (%) = 1−(absorbance at 520 nm of 0.15 mM DPPH plus test substance)/(absorbance at 520 nm of 0.15 mM DPPH)×100. From this experiment, the 50% DPPH radical scavenging concentrations (EC50) of the test substances were calculated. α-Tocopherol was used as positive control.

Cell Culture Rat heart-derived H9c2 cells were purchased from the American Tissue Culture Collection (Manassas, VA, U.S.A.). Cells were subcultured weekly in culture flasks containing 10 ml Dulbecco’s modified Eagle’s medium (Cambrex Bio Science, Walkersville, MD, U.S.A.) with 10% fetal bovine serum (Life Technologies, Rockville, MD, U.S.A.) and antibiotics (25 U/ml penicillin and 25 U/ml streptomycin). Cells were cultured (37 °C, 5% CO2) in 24-well plates containing 10 ml Dulbecco’s modified Eagle’s medium (V A, U.S.A.). Cells were subcultured weekly in culture flasks containing 10 ml Dulbecco’s modified Eagle’s medium (2 mM) for 2 h, by which time cell insult was assessed.

Comet assays were performed using a modification of the method described previously. Single Cell Gel Electrophoresis Assay (Comet Assay) Analysis Comet assays were performed using a modification of the method of Rojas et al. Briefly, H9c2 cells were embedded in a bed of 0.5% low-melting-point agarose with a mitochondrial-dependent reduction of MTT to formazan. The amount of formazan formed was quantified by measuring the absorbance of the solution at 540 nm. In addition, release of LDH in the culture medium was determined as an indicator of cell necrosis by spectrophotometric analysis at 340 nm as previously described. The MDA level in H9c2 was determined by commercial immunoassay kit (Bioxytech MDA-OxisResearch, Portland, Oregon, U.S.A.) according to the recommendations of the manufacturer. Protein concentration was determined by the Bradford method using bovine serum albumin as standard.

Single Cell Gel Electrophoresis Assay (Comet Assay) Analysis Comet assays were performed using a modification of the method of Rojas et al. Briefly, H9c2 cells were embedded in a bed of 0.5% low-melting-point agarose with a cell density of about 200—300 cells per sample. Aliquots were placed on microscope slides that had been previously coated with 1% normal agarose. After gel slides were immersed for 3 h in ice-chilled lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris pH 11.0, 10% DMSO, 1% Triton X-100), electrophoresis was conducted at 30 V, 250 mA for 7 min. The slides were washed with 1 mM ammonium acetate in 70% ethanol and 1 mg/ml spermine in 70% ethanol before staining with SYBR Green (Sigma-Aldrich Co., St. Louis, MO, U.S.A.), a DNA intercalating fluorescent dye. Fluorescent stained nucleotides were obtained using the imaging system of Zeiss, Germany. Fluorescent stained nucleotides were quantified using image analysis CASP program downloaded from http://www.casp.of.pl. The 15—20 images were randomly selected from each sample and comet tail moment was measured according to the procedure of Konca et al. The data shown represent the image analysis from three independent experiments.

Measurement of Vasorelaxation in Isolated Rat Aorta Thoracic aorta isolated from SD rats was cut into rings of 2—3 mm width with extreme care to preserve endothelium intact as previously reported. The aortic preparations were suspended in an organ bath containing 20 ml of Krebs’ bicarbonate buffer (mm: NaCl, 118; KCl, 4.7; CaCl2, 2.5; NaHCO3, 25; MgSO4, 1.2; KH2PO4, 1.2; and glucose, 11.0) bubbled with mixture gas (95% O2, 5% CO2) and maintained at 37 °C. The aortic preparations were allowed to equilibrate for 90 min under the resting tension of 2 g. The isometric contractile response was measured with a force displacement transducer (Grass FT03, Grass Ins., Quincy, MA, U.S.A.) and displayed on a chart recorder (Multicorder MC 6625, Hugo Sachs Electronic, March, Germany). The aortic preparations were precontracted submaximally with phenylephrine (0.3 μM). After the contraction was stabilized, acetylcholine (1 μM) was added to confirm the presence of the endothelium. Then, the aortic preparations were washed 3 times for 45 min, and rechallenged with phenylephrine. RBUM were cumulatively added to the tissue bath after phenylephrine (0.3 μM) response reached plateau. In separate experiments, endothelium-intact aortic preparations were pretreated with Nω-nitro-L-arginine methyl ester (10 μM), an inhibitor of nitric oxide synthase, 15 min prior to exposure to phenylephrine. In other sets of experiments, the endothelium-intact aortic preparations were precontracted by KCl (45 mM) or pretreated with the non-selective Ca2+-activated K+ (KCa) channel blocker, tetraethylammonium chloride (TEA, 10 mM), 45 min prior to exposure to phenylephrine.

Measurement of Blood Pressure and Heart Rate in SHRs Systolic blood pressure (SBP) and heart rate (HR) from SHRs were measured using the tail-cuff method as previously reported. Rat tails were occluded with an appropriate size tubular tail cuff (7/16 inch, 12 mm) connected to photoplethysmograph (Model 31, IITC Life Sci., Woodland Hills, CA, U.S.A.) and pulse was detected as the cuff pressure was lowered. To measure the blood pressure, rats were prewarmed at 32 °C for 5—10 min in a restraining cage in a warming box. Rats were allowed to habituate to this procedure for ≥2 weeks before experiments. SHRs with SBP >170 mmHg were used in this study. All rats were treated with oral administration of vehicle (1% arabia gum), RBUM (100 mg/kg, once a day), or captopril (30 mg/kg) for 42 d. In our preliminary studies, 10 or 30 mg/kg of RBUM hardly exerted antihypertensive activity. In addition, long-term treatment with higher doses of the extracts has a possibility to cause adverse effects such as hepatic toxicity. Thus the single dose of 100 mg/kg of RBUM was selected to measure the long-term antihypertensive effects. Old-matched WKY rats were used as control. SBP and HR were measured 2.5 h after oral administration of RBUM.

Evaluation of Vasorelaxant Activity in Aorta from SHRs After completing the previous antihypertension study in SHRs, the same animals were used for this in vitro experiment. On day 43 after repeated daily oral dosing of RBUM, the thoracic aorta was isolated from SHRs and a similar protocol as described in previous vasorelaxant study
was performed. Briefly, the relaxant effects of acetylcholine and sodium nitroprusside on endothelium-intact arteries from SHRs were analyzed in arteries contracted by phenylephrine (0.3 \( \mu \)M) and finally a cumulative concentration response to acetylcholine or sodium nitroprusside was obtained.

**Measurement of Blood Lactate Dehydrogenase and Hepatic Malondialdehyde Levels** On day 43 after repeated daily oral dosing of RBUM, blood samples were taken from the inferior vena cava of SHRs for determination of LDH. The liver from SHRs was rapidly isolated and frozen in liquid nitrogen for the determination of hepatic MDA as an index of lipid peroxidation. The frozen liver was transferred into 1:2 w/v in Tris buffer (20 mM, pH 7.2) and homogenized using an Ultra-Turrax (Ika® Works, Model T25 Basic, Japan). After homogenates were centrifuged at 4000\( \times g \) at 4 \( ^\circ \)C for 10 min, the supernatant was taken for the assays of MDA contents. The MDA level was determined using a commercial immunoassay kit (Bioxytech MDA-586, OxisResearch, Portland, OR, U.S.A.) according to the recommendations of the manufacturer.

**Chemicals and Solutions** Phenylephrine, acetylcholine, sodium nitroprusside, N\textsuperscript{G}-nitro-L-arginine methyl ester, and tetraethylammonium chloride were obtained from Sigma-Aldrich Co., and were dissolved in distilled water. Acetylcholine with high hygroscopicity was prepared at 10 mM in distilled water as stock solutions, which were subdivided into aliquots to use when needed without handling the total sample and stored at \(-20{\circ}\)C, each aliquot being used for each experiment with serial dilution. In addition, RBUM was dissolved in 100% dimethylsulfoxide and diluted with distilled water for *in vitro* study, and suspended in 1% arabia gum for *in vivo* study. All solutions were freshly prepared immediately before the experiments.

**Statistical Analysis** All values are expressed as mean±S.E.M., except values from the cell study, which are expressed as mean±S.D. Significant differences between responses were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett’s test for multiple comparisons (Sigma Stat, Jandel, San Rafael, CA, U.S.A.). In all comparisons, the difference was considered statistically significant at \( p<0.05 \).

**RESULTS**

**DPPH Radical Scavenging Activity** To determine whether RBUM had radical-scavenging activities, we measured its effect in scavenging DPPH radicals. As shown in Fig. 1, RBUM showed free radical scavenging activities in a concentration-dependent manner (5.2±1.5%, 39.5±1.3%, 77.5±0.15%, and 86.4±0.1% at 3, 10, 30, and 100 \( \mu \)g/ml, respectively), with an EC\(_{50}\) value 14.3 \( \mu \)g/ml. In comparison, \( \alpha \)-tocopherol as positive control was used and EC\(_{50}\) value obtained was 4.5 \( \mu \)g/ml.

**Protective Effect on Hydrogen Peroxide-Mediated Oxidative Insult in H9c2 Cells** The protective effects of RBUM on hydrogen peroxide-induced oxidative insult were assessed using MTT reduction and LDH release assays. As shown in Fig. 2A, after H9c2 cells were subjected to 2 mM hydrogen peroxide for 2 h, cell viability was reduced by approximately 75%. Pretreatment with RBUM (1—100 \( \mu \)g/ml) protected the H9c2 cells against hydrogen peroxide insult in a concentration-dependent manner (55.5±7.83% and 97.0±7.6% at 30 and 100 \( \mu \)g/ml, respectively). In addition, LDH leakage, an indicator of cell necrosis, was evaluated in the same condition. As shown in Fig. 2B, hydrogen peroxide induced increase of LDH leakage was markedly decreased by treatment with 3, 10, 30,
and 100 μg/ml RBUM (138.0±3.5, 121.3±12.2, 85.3±13.0, and 73.3±3.1 unit/ml, respectively). To confirm the antioxidant activity of RBUM, oxidative DNA damage (comet tail moment) and lipid peroxidation (MDA formation) were evaluated. As shown in Fig. 3A, hydrogen peroxide treatment markedly increased comet tail moment in the control group (64.0±17.5%) compared with that of the normal group (14.3±1.3%). This increase in comet tail moment was significantly decreased by pretreatment of RBUM (29.4±12.4% and 26.2±9.0% at 10 and 100 μg/ml, respectively). In evaluating lipid peroxidation (Fig. 3B), hydrogen peroxide overload provoked higher formation of MDA in the control group (3.54±0.99 μM) compared with that of the normal group (0.86±0.43 μM). Pretreatment with RBUM significantly suppressed MDA formation induced by hydrogen peroxide (2.36±0.62 and 1.88±0.91 μM at 10 and 100 μg/ml, respectively).

Vasorelaxant Effects on Isolated Rat Aortas  The vascular relaxant effects of RBUM were measured as the inhibition percentage of the contraction induced by pretreatment of phenylephrine (0.3 μM) in isolated rat aortic preparations. As shown in Fig. 4A, RBUM exhibited great vasorelaxant activity in phenylephrine-induced contraction of rat aorta in a concentration-dependent manner (EC50 value: 1.9±0.2 μM/ml). This vasorelaxant effect was completely abolished by denudation of the endothelial layer. Furthermore, pretreatment of aortic tissue with an effective nitric oxide synthase inhibitor, Nω-nitro-L-arginine methyl ester (10 μM), also significantly blocked RBUM-induced vasorelaxant effect. In a separate experiment, raising extracellular K+ (Kc+) channel blocker tetrodotoxin (10 μM) 45 min prior to exposure to phenylephrine. Results are expressed as means±S.E.M. (n=8—10). *p<0.05, significantly different from vehicle-treated group.

Antihypertensive Effects in SHRs  The effects of repeated oral administration with 100 mg/kg RBUM for 42 consecutive days on SBP and HR in SHRs are shown in Fig. 5. The basal values of SBP and HR in WKY rats were 142±3.6 mmHg and 352±8.7 beats/min (n=12), respectively. In contrast, the predose values of SBP and HR in
SHRs were 190 ± 2.6 mmHg and 411 ± 4.6 beats/min (n = 23), respectively. In vehicle-treated SHRs, SBP was slowly increased in a time-dependent manner (approximately 20 mmHg; Fig. 5A). RBUM (100 mg/kg, p.o.) decreased SBP (approximately 20 mmHg decrease compared with vehicle-treated group), although the difference was not statistically significant. In all groups, HR was not different compared with the vehicle-treated group (Fig. 5B).

**Vasorelaxant Effects on Aortas from SHRs Chronically Treated with RBUM**

The effects of acetylcholine and sodium nitroprusside were investigated on endothelium-intact aortic preparations from SHRs that were daily treated with RBUM (100 mg/kg) for 42 consecutive days. Both acetylcholine and sodium nitroprusside caused concentration-dependent relaxations of phenylephrine-precontracted aortic preparations from all groups of animals. Acetylcholine-induced relaxant response in aorta from vehicle-treated SHRs (E\text{max} = 43.3 ± 3.4%) was significantly decreased compared with that of WKY rats (E\text{max} = 77.7 ± 3.0%; Fig. 6A). This decrease in relaxation in response to acetylcholine (E\text{max} = 58.9 ± 3.4%) was significantly improved by treatment with RBUM extract. Moreover, the decrease of vasorelaxation induced by sodium nitroprusside in SHRs (E\text{max} = 77.1 ± 2.4%) as compared with that of WKY rats (E\text{max} = 97.1 ± 0.9%) was significantly recovered by treatment with RBUM (E\text{max} = 95.2 ± 1.4%; Fig. 6B).

**Effects on Oxidative Status in SHR**

Hepatic MDA was significantly increased in vehicle-treated SHRs (47.5 ± 2.6 nmol/g) compared with that of WKY rats (29.1 ± 0.9 nmol/g; Fig. 7A). This increase in liver lipid peroxidation was significantly reduced by long-term administration with 100 mg/kg RBUM (37.4 ± 1.9 nmol/g). LDH activity in the vehicle group...
(87.6±8.0 unit/ml) was significantly increased compared with that in WKY rats (51.0±3.8 unit/ml). This increase in LDH activity in plasma from SHR was reduced by long-term administration with RBUM (66.6±7.4 unit/ml), although not significantly.

DISCUSSION

The present study demonstrates that RBUM has potent protective effect in hydrogen peroxide-induced oxidative insult in H9c2 cells, vasorelaxant activity in isolated rat aortic preparations, and antihypertensive effect in SHRs.

Oxidative stress has been implicated in the formation and progression of a number of cardiovascular diseases including hypertension.\(^1\) Cardiovascular damage associated with hypertension can generate destructive oxidants and oxygen free radicals, which are very toxic to vessels and may result in further tissue necrosis and ROS-mediated cardiovascular remodeling.\(^2\) Thus removal of excess ROS or suppression of their generation by antioxidants may be effective in preventing oxidative cell death. In the present study, using various \textit{in vitro} experiments, we found that RBUM has potent free radical-scavenging activity in DPPH assay and has the ability to protect against hydrogen peroxide-induced oxidative insults in H9c2 cells. In addition, RBUM was shown to inhibit comet tail moment and MDA formation. These potent activities on oxidative DNA damage and lipid peroxidation are thought to reflect protective mechanisms of RBUM in hydrogen peroxide-induced oxidative insults.

In isolated aortic preparation, RBUM exhibited substantial direct vasodilator effect on phenylephrine-induced contraction of aortic preparations (EC\(_{50}\) value; 1.9 \(\mu\)g/ml). This vasorelaxation was completely blocked by removing functional endothelium or pretreatment with \(\text{N}^6\)-nitro-l-arginine methyl ester, a well known non-selective nitric oxide synthase inhibitor.\(^3\) These results suggest that the vasorelaxation caused by RBUM may be mediated by endothelium-dependent nitric oxide signaling pathway. In the study to investigate the role of potassium channels in RBUM-induced relaxations, decreasing the \(K^+\) gradient across the cell membrane by raising extracellular \(K^+\) (45 mM) had a marked inhibitory effect on RBUM-induced relaxation in endothelium-intact preparations, which suggests that RBUM may hyperpolarize aortic ring by increasing \(K^+\) conductance.\(^4\) Similar results were also obtained by pretreatment with the nonselective blocker of \(K_{ca}\) channel, tetraethylammonium chloride (1, 3, 10 mM). This concentration-dependent inhibition of relaxation caused by pretreatment with tetraethylammonium chloride suggests that the activation of \(K_{ca}\) channels might, at least in part, involve RBUM-induced relaxant responses.

In SHR study, we observed that repeated oral administration with RBUM (100 mg/kg) for 42 consecutive days induces substantial antihypertensive effect compared with that of vehicle-treated SHRs. SBP in SHRs treated with RBUM was decreased by approximately 20 mmHg (ca. 10%) compared with that of vehicle-treated SHRs. Another objective of the present study was to investigate whether long-term treatment with RBUM improves endothelial function of conduit arteries. It is known that structural and functional alterations of the vascular endothelium occur in much pathology, including arterial hypertension.\(^5\) These alterations are important in the mechanisms that determine blood pressure, the regression of which is generally regarded as an important target of antihypertensive therapy.\(^6\) We have observed that the diminished relaxation in response to acetylcholine and sodium nitroprusside in SHRs was significantly improved by long-term treatment with RBUM. These results suggest that long-term administration of RBUM may restore the impaired endothelial-dependent as well as -independent vascular dilation on aortic preparations from SHRs. We speculate that the antihypertensive effect of RBUM may also be owing to its recovery property against structural and functional alterations of the vascular endothelium in SHR. In addition, long-term treatment with RBUM significantly reduced lipid peroxidation (hepatic MDA) and LDH activity in plasma in SHRs. These results reflect that antioxidant property of RBUM could reduce oxidant status in SHR.

In conclusion, the results from the present study suggest that long-term administration of RBUM causes antihypertensive effects in SHRs, and its antihypertensive effect might be due to a combination of vasodilator and antioxidant actions of RBUM. In addition, the vasorelaxant effects of RBUM might be mediated, at least in part, through increase in release of nitric oxide and through activation of \(K_{ca}\) channels in vessels. However, further studies are necessary clearly to elucidate the underlying mechanisms responsible for these responses.

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