Antihypertensive Effects of the Methanol Extract of *Sorbus* Cortex in the Nitric Oxide-deficient Hypertensive Rat

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A pharmacological inhibition of nitric oxide synthase (NOS) in rats produces vasoconstriction, renal dysfunction, and hypertension. The present study was aimed at investigating whether the methanol extract of Sorbus commixta cortex (MSC) ameliorates N^G-nitro-L-arginine methylester (L-NAME) induced hypertension in rats. Treatment of rats with L-NAME (10 mg/kg/day in drinking water, 5 weeks) caused a sustained increase in systolic blood pressure (SBP). Administration of MSC (100 or 200 mg/kg/day, p.o) significantly lowered the SBP in the L-NAME-treated rats and this effect was maintained throughout the whole experimental period. Moreover, ecNOS expression in aorta and kidney tissue from L-NAME treated rats was significantly restored by administration of MSC. Furthermore, the impairment of acetylcholine (ACh)-induced relaxation of aortic rings in the L-NAME treated rats was reversed by administering of MSC. The renal functional parameters including urinary volume, sodium excretion, and creatinine clearance (Ccr) were also restored by administering MSC. Taken together, the present study suggeststhat MSC prevents the increase in SBP in rats with L-NAME-induced hypertension, which may result from the up-regulation of the vascular and renal ecNOS/NO system.

Key words : L-NAME, methanol extract of *Sorbus commixta* cortex (MSC), hypertension, vascular tone

Introduction

It has been demonstrated that the endothelium plays a fundamental role in determining vascular tone and mediates smooth muscle relaxation through a NO-cGMP pathway[1]. Endothelium-dependent relaxations are impaired and the ability of nitric oxide (NO) to maintain vascular tone is deficient in the hypertensive condition[2-4]. The synthesis of NO is mediated by NO synthases (NOS), a family of at least three isozymes that synthesize NO from the terminal guanidine nitrogen of L-arginine. NOS type I (neuronal or nNOS) and NOS type III (endothelial or ecNOS) are constitutively expressed, but quiescent until activated by increased Ca^{2+} levels that sustain calmodulin binding[5]. In contrast, NOS type II (inducible or iNOS) is strongly expressed after transcriptional activation by cytokines or lipopolysaccharides (LPS), the major component of bacterial endotoxin[6]. NO synthesis is completely inhibited by L-arginine derivatives such as N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine methyl ester (L-NAME), and others[7]. Pharmacological long-term blockade of NO synthesis by the chronic administration of an inhibitor of NO synthase (NOS) produces systemic arterial hypertension, vascular structural changes, and renal dysfunction. Accordingly, a vasodilator that was able to restore the level of NO in the vascular system could be used as a therapeutic agent for the treatment of hypertension. In fact, many studies have been performed with natural resources to find vasodilators for the treatment of hypertension and atherosclerosis[8-14]. Extracts from several natural resources that are used in Oriental medicine have already been shown to have an antihypertensive effect in NO-deficient hypetensive rats[15-17].

In a recent study, we found that a methanol extract from the *Sorbus commixta* (Malaceae) cortex (MSC) dilates vascular smooth muscle via endothelium-dependent NO/cGMP signaling and protects the development of atherosclerosis[18,19]. Such an effect may be beneficial in treating human hypertension. Unfortunately, information regarding the in vivo effects of MSC on hypertension is scarce. The present study, therefore, was aimed at investigating the effects of chronic MSC treatment on blood pressure in a rat model of L-NAME-induced hypertension.

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· Received : 2005/11/29 · Revised : 2006/01/09 · Accepted : 2006/02/02

Materials and Methods

1. Plant material and Extraction

The stem bark of *S. commixta* (Malaceae) was purchased from the herbal medicine co-operative association of Junbuk Province, Korea, in October 2003. A voucher specimen (No. BDR 20) was deposited in the Herbarium of the Professional Graduate School of Oriental Medicine, Wonkwang University (Korea). The S. commixta (1.0 kg) was air-dried at room temperature and reduced to a fine powder by milling. The powder was subjected to extraction with 800 ml of methanol, three times, 24 hours each. The methanolextract was filtered through Whatman No. 3 filter paper and concentrated using a rotary evaporator (61.2 g).

2. Animals

All procedures for animal treatment were carried out in accordance with theNational Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and were approved by the Institutional Animal Care and Utilization Committee for Medical Science of Wonkwang University. Male Sprague-Dawley rats (weighing 170 to 200 g) were purchased from Korean Experimental Animals Co. (Daejeon, Korea). To collect 24-hr urine samples, the rats were housed in metabolic cages in an animal room with an automatic temperature (22 ℃) and lighting (12 hr light-dark cycle) control. A 1-week adaptation period for vehicle (tap water) administration and blood pressure measurements was allowed before the experimental protocol was initiated. Over the course of 5 weeks, the experimental protocol was carried out. During the first two weeks, the rats were allocated a normal rat chow diet with water alone (control group) or with water containing L-NAME (L-NAME group). The rats were then divided into five groups. For the remaining 3 weeks, the rats were treated as follows: 1) control group; 2) MSC-treated (200 mg/kg/day) control group; 3) L-NAME group (10 mg/kg); 4) MSC-treated (100 mg/kg/day) L-NAME group; 5) MSC-treated (200 mg/kg/day) L-NAME group (n=8, each group). Systolic blood pressure (SBP) was measured weekly in a conscious rats by tail-cuff plethysmography. At least six determinations were made in every session and the mean of the lowest three values, within 5 mmHg, was taken as the SBP. On the day of sacrifice, blood was collected immediately following decapitation for the measurement of sodium and potassium ion levels, osmolality, and creatinine concentration in the plasma. The thoracic aorta and kidneys were removed, immediately frozen in liquid nitrogen, and stored at -72 ℃ until assayed.

3. Monitoring of renal function

Each group was maintained in separate metabolic cages for 2 days, allowing quantitative urine collections and water intake measurements. 24-hr urine samples were collected (between 09:00 and 10:00 a.m) to determine the levels of creatinine, sodium, potassium, osmolality, and other parameters of renal function. Plasma osmolality and creatinine levels were also measured. Ion concentrations were measured using an electrolyte analyzer (NOVA 5, Biochemical, Waltmam, MA, USA). Osmolality was measured using an Advanced CRYOMATICTM osmometer (Model 3900, Advanced Instruments Inc., Norwood, MS, USA). Creatinine concentrations (Ccr) in the plasma and urine samples were measured with a colorimetric method using a spectrophotometer (Milton Roy, Rochester, NY, USA). Solute-free water reabsorption (TcH_2O) was calculated by the following formula: TcH_2O = V (Uosmol/Posmol - 1), where V is urine volume, Uosmol is urinary osmolality, and Posmol is plasma osmolality.

4. Preparation of aorta and recording of isometric vascular tone

The thoracic aortae were rapidly and carefully dissected from rats in 4 experimental groups and placed into ice-cold Krebs solutions (pH 7.4) containing 118 mmol/L NaCl, 4.7 mmol/L KCl, 1.1 mmol/L $MgSO_4$, 1.2 mmol/L KH_2PO_4, 1.5 mmol/L $CaCl_2$, 25 mmol/L $NaHCO_3$, and 10 mmol/L Glucose. The aortae were cleared free of connective tissue and fat, and then cut into rings with a width of approximately 3 mm. All dissecting procedures were done with extreme care to protect the endothelium from inadvertent damage. The aortic rings were suspended by means of two L-shaped stainless steel wires inserted into their lumens and placed in a tissue bath containing Krebs solution (pH 7.4) at 37 ℃. 95% O_2-5% CO_2 was continuously bubbled through the bath. The baseline load placed on the aortic rings was 2.0 g, and the changes in isometric tension were recorded using a force-displacement transducer (Grass FT 03, Quincy, MA, USA) connected to a Grass polygraph recording system (Model 7E). In the first set of experiments, the aortic rings were contracted with phenylephrine (3×10^{-6} M) to obtain their maximal response. The aortic rings were then washed every 20 min with Krebs solution until their tension returned to the basal level. A concentration-dependent response curve to acetylcholine (ACh) (10^{-9} - 10^{-5} M) was determined and used as a positive control to verify endothelium-intact aortic rings, contracted by 3×10^{-6} M phenylephrine. The effect of vehicle, < 0.2% dimethylsulfoxide (DMSO), was also tested. After each test, the aortic rings were washed three times with fresh Krebs solution and allowed to equilibrate for 30 min. Endothelium-dependent

and endothelium -independent aortic relaxation was carried out by the cumulative addition of acetycholine chloride (ACh) and sodium nitroprucide (SNP), respectively. After each test, the aortic rings were washed three times with fresh Krebs solution and allowed to equilibrate for 30 min.

5. Protein preparation and Western blot analysis

The thoracic aortae and kidneys were homogenized in solutions containing 250 mmol/L sucrose, 1 mmol/L EDTA, 0.1 mmo/L phenylmethylsulfonyl fluoride (PMSF) and 20 mmol/L potassium phosphate buffer, at pH 7.6 with a Polytron homogenizer at 3,000 rpm. Large tissue debris and nuclear fragments were removed by two low speed spins in succession (1,000 g, 5 min; 10,000 g 10 min) at 4 ℃. Supernatants from these low speed spins were ultracentrifuged at 100,000 g for 1 hour at 4 ℃. Pellets were resuspended for protein blotting and the protein concentrations were determined by the Bradford method[20] with bovine serum albumin as a standard. Protein samples (50 μg) were electrophoretically fractionated with a discontinuous system consisting of a 8% polyacrylamide resolving gel and a 5% stacking gel, followed by transfer to a nitrocellulose membrane at 20 V and 100 mA (current constant) overnight. The membrane was washed, blocked, and then incubated with primary antibodies (1:2000 dilutions) against ecNOS (Transduction Laboratories, Lexington, KY, USA). The bound horseradish peroxidase-conjugated secondary antibody was detected with an enhanced chemiluminescence (Amersham, Buckingghamshire, England) procedure. Protein expression levels were determined by analyzing the signals captured on the nitrocellulose membrane (Amersham, Buckinghamshire, England) using a Chemi-doc image analyzer (Bio-Rad, Hercules, CA, USA).

6. Statistical analysis

Relaxant responses are expressed as percentage relaxation from phenylephrine pre-contraction levels. Results were expressed as means ± SEM. The statistical significance of the difference between the group means was determined using the one-way ANOVA and Student's t-test.

Results

1. Effect of MSC on SBP

Fig. 1 shows SBPs for the five experimental groups over the experimental period. When the experiment began, SBPs for the five experimental groups were approximately 96-108 mmHg. After one week, SBPs for the L-NAME treated rats increased to 125 ± 3.9 mmHg. After two weeks, SBPs for the L-NAME treated rats reached a plateau (148 ± 4.2 mmHg, $p<0.01$ vs. 112 ± 2.9 mmHg), which was maintained thereafter. Treatment with MSC (1-dose; 100 mg/kg/day or 2-doses; 200 mg/kg/day) significantly lowered the elevated SBPs for the L-NAME treated rats. This effect was maintained throughout the experimental period. There were no significant differences in SBPs between the control and MSC-treated control groups during the experimental period.

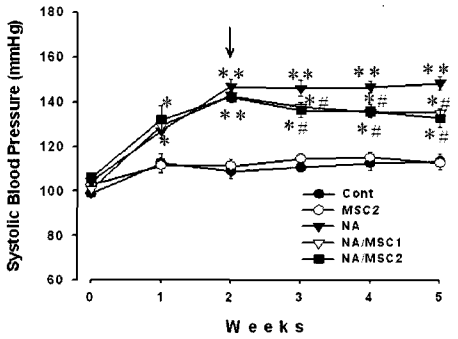

Fig. 1. Effects of MSC on systolic blood pressure in L-NAME-induced hypertensive rats. Cont: normal group, MSC2: MSC-treated (200 mg/kg/day) control group, NA: L-NAME group (10 mg/kg/day), NA/MSC1: MSC-treated (100 mg/kg/day) L-NAME group, NA/MSC2: MSC-treated (200 mg/kg/day) L-NAME group. *p(0.05, **p(0.01 compared with control; #p(0.05, compared with L-NAME-treated group. Arrow indicates administration of MSC.

2. Effect of MSC on the expression level of ecNOS in the kidney and aorta

As shown in Fig. 2, the expression level of ecNOS in the aorta was significantly decreased in L-NAME-treated rats ($p<0.01$ vs. control group). This down regulation of ecNOS expression in the aortae of L-NAME-induced hypertensive rats was corrected by co-administrating MSC ($p<0.05$ vs. L-NAME-treated group).

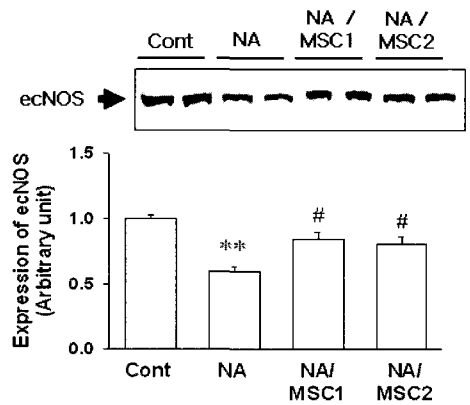

Fig. 2. Effect of MSC on the expression level of endothelial nitric oxide synthase (ecNOS) in the aorta. Representative Western blot and densitometric analysis of the ecNOS expression in the aorta are shown. See Fig. 1 for other legends. *p(0.01 compared with control; #p(0.05, compared with L-NAME-treated group.

Fig. 3 shows that the expression level of ecNOS in the kidneys of L-NAME-treated rats was significantly attenuated compared with that of the control group ($p<0.01$ vs. control group). Co-administrating MSC significantly reversed the

attenuation of the ecNOS expression level in the kidneys of L-NAME-treated rats ($p<0.05$ vs. L-NAME-treated group).

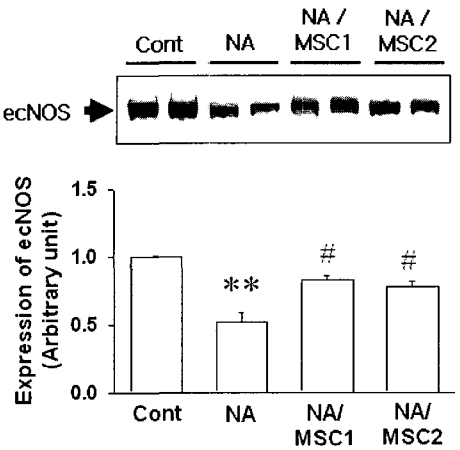

Fig. 3. Effect of MSC on the expression level of endothelial nitric oxide synthase (ecNOS) in the kidney. Representative Western blot and densitometric analysis of the ecNOS expression in the whole kidney ar·1e shown. See Fig. 1 for other legends. **p(0.01, compared with control; #p(0.05, compared with L-NAME-treated group.

3. Effect of MSC on vascular tone

The endothelial function of the aorta was determined by measuring its vascular response to ACh and SNP. The endothelium-dependent relaxant response to ACh was significantly reduced in the aorta from L-NAME-treated rats (Fig. 4A). Treatment with MSC reduced the impairment to ACh-stimulated relaxation in L-NAME-treated rats (Fig. 4A). The endothelium-dependent relaxant response to SNP was not reduced in the aorta from L-NAME-treated rats (Fig. 4B). Therefore, treatment with MSC did not alter the SNP-induced vascular relaxant responsein the L-NAME-treated rats (Fig. 4B).

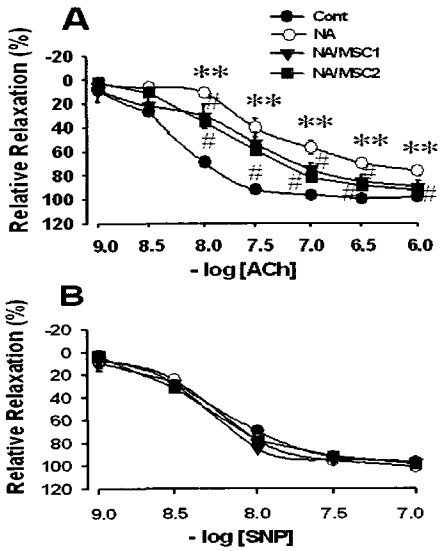

Fig. 4. Effect of MSC on vascular tone. Endothelium-dependent or independent relaxation response to cumulative doses of ACh (A) or SNP (B) in rat aortic segments isolatedfrom the experimental groups was examined. See Fig. 1 for other legends. Values are mean ± SEM (n=12, each group). **p(0.01 vs. control group; #p(0.05 vs. L-NAME group.

4. Effects of MSC on renal function

Table 1 contains the final body weights and renal functional parameters for the experimental groups at the end of the experimental period. During the 5-week experiment, there was no difference in body weights among the five experimental groups. However, administering L-NAME for 5 weeks significantly lowered renal creatinine clearance (Ccr), urinary flow rate (UV), and sodium excretion (UNaV) ($p<0.05$ vs. control, each). These changes were fully reversed by supplementation with MSC during the last 3 weeks ($p<0.05$ vs. L-NAME-treated group). Renal potassium (UKV) and chloride (UClV) excretion and solute-free water reabsorption (TcH_2O) were not altered in the L-NAME-treated group.

Table 1. Effects of MSC on body weight and renal function in rats with L-NAME- induced hypertension

	Experimental Groups				
	Cont	Cont/MSC2	NA	NA/MSC1	NA/MSC2
BW(g)	365.6±3.1	358.6±7.2	354.2±9.2	343.5±6.4	344.2±10.6
UV(ml/kg/day)	28.1±1.9	29.9±2.1	24.4±1.2*	29.3±1.7#	28.6±1.2#
UNaV(Eq/min/kg)	2.55±0.26	2.44±0.21	2.07±0.19*	2.69±0.21#	2.74±0.27#
UKV(Eq/min/kg)	4.78±0.17	4.97±0.30	4.14±0.40	4.74±0.19	4.91±0.26
UClV(Eq/min/kg)	5.99±0.30	6.04±0.34	5.73±0.81	6.39±0.18	6.78±0.43
Ccr(μl/min/kg)	2.54±0.14	2.67±0.16	2.13±0.18*	2.52±0.12#	2.47±0.22#
TcH_2O(μl/min/kg)	189.5±6.3	181.1±7.3	165.4±14.7	182.3±7.0	179.5±13.6

Cont: normal group, Cont/MSC: MSC-treated (200 mg/kg/day) control group, NA: L-NAME group (10 mg/kg/day), NA/MSC1: MSC-treated L-NAME group, NA/MSC2: MSC-treated (200 mg/kg/day) L-NAME group. BW: body weight, UV: urinary volume, UNaV: urinary sodium excretion, UKV: urinary potassium excretion, UClV: urinary chloride excretion, Ccr: creatinine clearance, TcH_2O: solute-free water reabsorption. Values are mean±SEM (n=8, each group). *p(0.05, compared with control; #p(0.05, compared with L-NAME-treated group.

Discussion

While conducting an in vitro screen of various medicinal plant extracts, the methanol extract of Sorbus commixta cortex (MSC) was found to exhibit a distinct vasorelaxant activity via the activation of a vascular NO-cGMP pathway. The aim of this study was to investigate the antihypertensive effects of MSC in NO-deficient hypertensive rats. As early as the first week, the SBPs of Sprague-Dawley rats were significantly increased by orally administering L-NAME. This effect was sustained during the whole experimental period, which is in accordance with a previous study[21]. The ecNOS expression levels in the kidney and aorta weresignificantly attenuated in rats with L-NAME-induced hypertension compared to control rats. ACh-induced aortic vasorelaxation was impaired in L-NAME-induced hypertensive rats, while there was no significant difference inSNP-induced aortic vasorelaxation among the experiment groups. These findings most likely reflect the diminished action of endogenous NO as a vasodilator. L-NAME may have interfered with the expression of and/or enzymatic activity of NOS. It has been proposed that various forms of hypertension are characterized by

dysfunctional endothelium. It has been suggested that a deficient production of endothelium-derived NO results in diminished vasodilator tone. This would allow the vascular resistance to increase, and thereby, would contribute to the elevated BP[22]. In vitro observations of isolated vessels in a number of experimental models, including 2K1C (Goldbaltt) renovascular hypertensive rats, rats with aortic contraction, Dahl salt-sensitive rats, and rats with deoxycorticisterone acetate (DOCA)-salt hypertension, as well as spontaneously hypertensive rats, have demonstrated that endothelium-dependent vasorelaxation is impaired[2-4]. Similar abnormalities in endothelial integrity have been reported in vivo both on spontaneously hypertensive rats and in humans with essential hypertension[23,24]. These reports suggest that the endothelial dysfunction contributes to the hypertension because of a reduced production of NO. In the present study, MSC was shown to dilate vascular smooth muscle via endothelium-dependent nitric oxide-cGMP signaling. Based on these results, we proposed that the blood pressure in rats with L-NAME-induced hypertension could be lowered by treatment with MSC. The present data shows that administering of MSC significantly attenuates the SBP in the L-NAME-induced hypertensive rats and that the attenuation is associated with the restored aortic expression of ecNOS. The present study also shows that administering of MSC reverses the impaired vascular relaxation induced by ACh. On the other hand, in response to another NO donor, SNP, aortic relaxation is not attenuated in rats with L-NAME-induced hypertension. Therefore, no difference is noted in the aortic relaxation in L-NAME-induced hypertensive rats treated with or without MSC. Our findings also suggest that chronic treatment with MSC could be beneficial in lowering blood pressure in the NO-deficient hypertensive model via the upregulation of the vascular ecNOS system.

It is well known that NO is an important regulator of renal hemodynamics and sodium handling. In normal rats, the nitric oxide level increases as an adaptive response to increased dietary salt intake, perhaps facilitating natriuresis and thus blood pressure homeostasis[25]. On the contrary, animals treated with NOS inhibitors develop hypertension, with a resetting of the pressure-natriuresis relation toward a higher blood pressure[26]. In the present study, renal functional parameters including creatinine clearance (Ccr), urine volume (UV), and urinary sodium excretion rate (UNaV) were significantly lowered in the L-NAME-induced hypertensive rats without affecting urinary potassium and chloride excretion rates and solute-free water reabsorption (TcH_2O). The expression level of renal ecNOS was also attenuated in the L-NAME-induced hypertensive rats. Treatment of L-NAME-induced hypertensive rats with either low or high doses of MSC restored the impaired renal functional parameters in concomitance with the upregulation of therenal ecNOS expression level. These results suggest that MSC restored the renal functional defect in the L-NAME induced hypertensive rats by upregulating renal ecNOS expression.

In conclusion, the present study suggests that MSC prevents an increase in SBP in rats with L-NAME-induced hypertension, that this effect may be causally related with the up-regulation of vascular and renal ecNOS expression and, that this effect results in the restoration of vascular tone and renal function.

Acknowledgements

This study was supported by the grants of the Oriental Medicine R&D Project, Ministry of Health & Welfare, Republic of Korea (03-PJ9-PG6-SO02-0001).

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