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Water extract of *Zanthoxylum piperitum* induces vascular relaxation via endothelium-dependent NO-cGMP signaling

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ABSTRACT

Aim of the study: The aim of the present study was to define the effects of extracts of leaves of *Zanthoxylum piperitum* (ZP) on the vascular tension and its mechanisms responsible in rat thoracic aortic rings.
Materials and methods: Methanol extract of ZP and aqueous fraction of the methanol extract (AZP) were examined for their vascular relaxant effects in isolated phenylephrine-precontracted aortic rings.
Results: Methanol extract of ZP and aqueous fraction of the methanol extract (AZP) induced relaxation of the phenylephrine-precontracted aortic rings in a concentration-dependent manner. Endothelium-denudation abolished the AZP-induced vasorelaxation. Pretreatment of the endothelium-intact aortic rings with N^G-nitro-L-arginine methylester (L-NAME) and 1H-[1,2,4]-oxadiazolo-[4,3-α]-quinoxalin-1-one (ODQ) inhibited the AZP-induced vasorelaxation. Inhibition of Ca^{2+} entry via L-type Ca^{2+} channels failed to block the AZP-induced vasorelaxation. Extracellular Ca^{2+} depletion slightly but significantly attenuated the AZP-induced vasorelaxation. Thapsigargin significantly attenuated the AZP-induced vasorelaxation. Further, Gd^{3+} and 2-aminoethyl diphenylborinate (2-APB), inhibitors of store-operated Ca^{2+} entry (SOCE), markedly attenuated the AZP-induced vasorelaxation. Also, wortmannin, an inhibitor of Akt, an upstream signaling molecule of eNOS, attenuated the AZP-induced vasorelaxation. AZP increased cGMP levels of the aortic rings in a concentration-dependent manner and the effect was blocked by L-NAME, ODQ, thapsigargin, Gd^{3+}, 2-APB, and wortmannin. K^+ channel inhibition with glibenclamide and tetraethylammonium, cyclooxygenase inhibition with indomethacin, and adrenergic and muscarinic receptors blockade had no effects on the AZP-induced vasorelaxation.
Conclusion: Taken together, the present study suggests that AZP relaxes vascular smooth muscle via endothelium-dependent activation of NO-cGMP signaling through the Akt- and SOCE-eNOS pathways.
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1. Introduction

Vascular dysfunction is closely related to the high blood pressure. Maintenance of vascular homeostasis is one of the targets for the control of high blood pressure. Endothelial cells are an intimate modulator for the control of vascular homeostasis. Endothelial cells respond to humoral and physical stimuli by releasing endothelium-dependent vasodilators including endothelium-derived relaxing factor (Furchgott and Zawadzki, 1980) and prostacyclin (Jaffe, 1985). Nitric oxide (NO) is endothelium-derived relaxing factor (Palmer et al., 1987; Ignarro et al., 1987). Endothelial synthesis of NO is a main pathway controlling vascular function through the generation of cGMP in the vascular smooth muscle cells. NO derived from L-arginine by a family of NO synthase (NOS), endothelial (e), neuronal or inducible NOS, is involved in the regulation of physiological and pathophysiological functions. NO generation by eNOS depends on Ca^{2+}-dependant and also Ca^{2+}-independent signaling pathways (Flemming and Busse, 1999). NO activation of the soluble guanylyl cyclase (sGC) results in an increase of intracellular cGMP. The cGMP elicits vascular relaxation via cGMP-dependent protein kinase signaling.

Recently, we found extracts of *Zanthoxylum piperitum* eliciting a vasorelaxant effect in the course of searching for potential antihypertensive agents from the natural products. *Zanthoxylum piperitum* is deciduous shrubs of Rutaceae and plants native to the China, Japan and Korea. *Zanthoxylum piperitum* is commonly called Szechuan pepper and is used as a seasoning spice and traditional herbal medicine in Asia (Bryant and Mezine, 1999; Jiang and Kubota, 2001). *Zanthoxylum piperitum* has long been used as

Abbreviations: NO, nitric oxide synthase; PE, phenylephrine; cGMP, guanosine 3′,5′-cyclic monophosphate; sGC, soluble guanylyl cyclase; L-NAME, N^G-nitro-L-arginine methylester; ODQ, 1H-[1,2,4]-oxadiazolo-[4,3-α]-quinoxalin-1-one; 2-APB, 2-aminoethyl diphenylborinate; SOCE, store-operated Ca^{2+} entry; NOS, nitric oxide synthase; IBMX, 3-isobutyl-1-methylxanthine; SERCA, sarco- and endoplasmic reticulum Ca^{2+} ATPase; K_{ATP}, ATP-sensitive K^+ channels; Gd^{3+}, gadolinium chloride.

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a traditional Korean herbal medicine for the facilitation of blood circulation and control of diarrhea and stomachache (Lee and Kim, 2009; Kim et al., 2002). However, to the best of our knowledge, the effect of *Zanthoxylum piperitum* on the vascular tone has not yet been defined. The purpose of the present study was to define the vascular relaxant activity of water partition of methanol extract of leaves of *Zanthoxylum piperitum* and its mechanisms of vasorelaxation.

2. Materials and methods

2.1. Extraction of Zanthoxylum piperitum

The dried leaves of *Zanthoxylum piperitum* A.P. DC (ZP) was purchased from the Herbal Medicine Bunjigol Farm (Goesan-gun, Chungbuk, Korea), in October 2007. A voucher specimen *Zanthoxylum piperitum* A.P. DC (No. DH 121) was authenticated by professor Tae Oh Kwon, College of Life Science and Natural Resources, Wonkwang University, and deposited at the Herbarium of the Professional Graduate School of Oriental Medicine, Wonkwang University, Iksan, Jeonbuk, Republic of Korea. The leaves (250 g) were subjected to extraction with 8 L of methanol for a week. The methanol extract of *Zanthoxylum piperitum* (MZP) was evaporated (46.2 g) and resuspended in H_2O, and sequentially partitioned with H_2O (AZP, 25.3 g), n-hexane (HZP, 6.5 g), ethyl acetate (EZP, 6 g), and n-butanol (BZP, 3.1 g).

2.2. Preparation of rat aorta

The animal procedures were in strict accordance with the National Institute of Health Guidelines 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 of Wonkwang University. Male Sprague–Dawley rats were purchased from Korean Experimental Animals Co. (Daejeon, Korea). The rats (weighing 250–300 g) were sacrificed by decapitation. The thoracic aorta was rapidly and carefully dissected and placed into ice-cold Krebs solution (pH 7.4) containing (in mM) 118.0 NaCl, 4.7 KCl, 1.1 $MgSO_4$, 1.2 KH_2PO_4, 1.5 $CaCl_2$, 25.0 $NaHCO_3$, and 10.0 glucose. The aorta was removed free of connective tissue and fat, and then cut into rings of approximately 3 mm width. All dissecting procedures were done with extreme care to protect the endothelium from inadvertent damage. In some aortic rings, the endothelial layer was mechanically removed by gently rubbing the luminal surface of the aortic ring back and forth several times with plastic tubing. Endothelial integrity or functional removal was verified by the presence or absence of the relaxant response to acetylcholine (1 μM) on the phenylephrine (1 μM) contracted vessels.

2.3. Record of isometric vascular tone

The aortic rings were suspended by means of two L-shaped stainless-steel wires inserted into lumen in a tissue bath containing Krebs solution (pH 7.4) at 37 °C, while being continuously bubbled with 95(O_2–5(CO_2. The tissues were then allowed to equilibrate for 60 min. The baseline load placed on the aortic rings was 1.0 g, and the changes in isometric tension were recorded via a transducer (Grass FT 03, Grass Instrument Co., Quincy, MA, USA) connected to a Grass Polygraph recording system (Model 7E, Grass Instrument Co., Quincy, MA, USA). In the first series of experiments, the effects of ZP on the vascular tension were defined. The aortic rings were contracted with phenylephrine (1 μM) to obtain maximal response. Once the maximal response to phenylephrine had been obtained, the aortic rings were exposed to cumulative doses of testing agent and the responses were recorded. The responses were stopped by washing the aortic rings with fresh Krebs solution. To define the mechanisms by which AZP relaxes vascular smooth muscle, another series of experiments have been done in aortic rings. The aortic rings were exposed to various modulating agents for 20 min, and then aortic vasorelaxation was carried out by cumulative addition of AZP. After each test, the aortic rings were washed three times with fresh Krebs solution and allowed for 30 min to equilibrate. In the extracellular Ca^{2+}-depleted experiments, aortic rings were washed with Ca^{2+}-omitted solution four times for 40 min before the addition of phenylephrine and testing agents.

2.4. Measurement of cGMP levels in aortic rings

After equilibration of the aortic rings for 60 min in 5 ml of Krebs solution gassed with 95% O_2–5% CO_2 at a constant temperature shaking water bath (37 °C), rings were incubated for an additional 5 min in the presence of 3-isobutyl-1-methylxanthine (IBMX, 100 μM) before addition of phenylephrine. After the aortic rings were subjected to partitioned extract of ZP (AZP) in the presence and absence of modulators for 4 min, reactions were stopped by freezing the tissues at liquefied N_2. The tissues were weighed and homogenized with Polytron homogenizer in 6(trichloroacetic acid solution. The homogenates were centrifuged at 13,000 rpm for 15 min and supernatant was extracted three times with water-saturated diethylether, and sequentially concentrated with Speed-vac concentrator (Savant Instrument, Farmingdale, NY, USA), and the precipitates were resuspended with 20 mM Tris–HCl buffer (pH 7.4). The protein concentration was determined by the method of Bradford (1976). cGMP content was measured by the equilibrated radioimmunoassay as described previously (Kim et al., 1998). In brief, standards or samples were introduced in a final volume of 100 μl of 50 mM sodium acetate buffer (pH 4.8). Then, 100 μl diluted cGMP antiserum (Calbiochem-Novabiochem Co., San Diego, CA, USA) and iodinated cGMP (10,000 cpm/100 μl) were added in succession and incubated for 24 h at 4 °C. The bound form was separated from the free form by charcoal suspension. Results were expressed as picomoles of cGMP per milligram of protein per minute.

2.5. Reagents

Acetylcholine chloride, phenylephrine HCl, N^G-nitroarginine methyl ester (L-NAME), 1H-[1,2,4]-oxadiazolo-[4,3-α]-quinoxalin-1-one (ODQ), (±)-verapamil HCl, indomethacin, glibenclamide, tetraethylammonium chloride (TEA), 3-isobutyl-1-methylxanthine (IBMX), atropine, and (±)-propranolol HCl were purchased from Sigma Chemical Co (St. Louis, MO, USA). Wortmannin, gadolinium chloride and 2-aminoethyl diphenylborinate (2-APB) were purchased from Biomol (Plymouth Meeting, PA, USA). Thapsigargin was purchased from Alomone (Jerusalem, Israel).

2.6. Statistical analysis

Relaxant responses are expressed as a percentage relaxation of the phenylephine (1 μM) precontraction levels unless otherwise described in the figure legends. Results were expressed as mean ± SEM. The statistical significance of difference between the group means was determined using one-way ANOVA and Student's *t*-test.

3. Results

3.1. Effects of ZP extracts on the vascular tone of aortic rings

With endothelium-intact aortic preparations the MZP and partitions of the MZP relaxed phenylephrine-precontracted aortic

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