Vasodilatory Effect of Wogonin on the Rat Aorta and Its Mechanism Study

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Wogonin, a natural flavonoid, is one of the bioactive compounds of the medicinal herb *Eucommia ulmoides* Oliv., widely used in southeastern Asia for treating hypertension. However, the molecular mechanisms for the therapeutic benefits remain largely unclear. The present study investigated the vasodilatory effect of wogonin and its possible mechanisms. The flavonoid (0.1–100 μM) caused concentration-dependent relaxations in endothelium-intact aortic rings precontracted with norepinephrine (NE, 1 μM) or potassium chloride (KCl, 60 mM). Preincubation with wogonin (10, 100 μM) for 20 min significantly inhibited the contractile responses to NE (0.1, 1, 10 μM) or KCl (7.5, 15, 30, 60 mM). Relaxant responses to wogonin were not inhibited by Nω-nitro-L-arginine methyl ester (100 μM) or endothelial denudation. In a Ca²⁺-free Krebs’ solution, wogonin not only blocked Ca²⁺ influx-dependent vasoconstriction by either NE (1 μM) or KCl (100 mM), but also inhibited NE (1 μM)-induced tonic contraction, which is dependent on intracellular Ca²⁺ release. Wogonin also suppressed the elevation of [Ca²⁺], induced by KCl (60 mM) after exhausting the calcium store in sarcoplasmic and endoplasmic reticula with thapsigargin (1 μM) or by ATP (100 μM) in primary vascular smooth muscle cells. These findings suggest that wogonin-induced responses are mainly due to the inhibition of both intracellular Ca²⁺ release and extracellular Ca²⁺ influx.

**Key words** wogonin; vasodilation; vascular smooth muscle cell; calcium ion

*Eucommia ulmoides* Oliv. bark, as a tonic herb, has been widely used in traditional herbal prescriptions in China, especially for treating hypertension. Experiments in vivo and in vitro have shown that different compounds contained in *Eucommia ulmoides* Oliv. bark exerted anti-hypertensive activities. Geniposidic acid and genipin could reduce the blood pressures of hypertensive rats. Caffeic acid could induce the nitric-oxide (NO) synthase in endotheliocyte. Besides, liriodendrin, (+)-syringaresinol and (+)-pinoresinol di-O-β-D-glucopyranoside could inhibit the cAMP activity. These compounds might contribute to the anti-hypertensive effect collectively. However, the anti-hypertensive effect of *Eucommia ulmoides* Oliv. bark remains largely unclear.

Pharmacological studies have revealed that *Eucommia ulmoides* Oliv. bark extract induce endothelium and NO-cyclic guanosine monophosphate (cGMP) dependent relaxation in the rat thoracic aorta. Another report demonstrated that the endothelium-dependent vascular relaxation induced by the bark extract is mediated by NO and endothelium-derived hyperpolarizing factor in small vessels. However, the vasorelaxing components have been unclear. Recently, we found oroxylin A and wogonin isolated from *Eucommia ulmoides* Oliv. bark could significantly lower the perfusion pressure. In the previous study, we had reported that oroxylin A could relax rat thoracic aorta and it was endothelium and NO dependent. The present study was undertaken to investigate vasodilatory effect of wogonin and its mechanism.

Wogonin (Fig. 1) is a flavone and has a variety of cardiovascular protective effect. It could regulate migration, proliferation and apoptosis of vascular smooth muscle cells. Besides, wogonin could inhibit angiogenesis, suppress collagen deposition in cardiac fibroblasts and inhibit ischemic brain injury. There is no evidence for vascular relaxation effect of wogonin. We describe here that wogonin, unlike oroxylin A, is an endothelium- and NO-independent vasodilatory flavonoid. One report demonstrated that wogonin offered a wide margin of safety. It has therapeutic potential for the treatment of cardiovascular and cerebrovascular diseases. However, it was reported that little wogonin was detected in...
MATERIALS AND METHODS

Reagents The following drugs were used: NaCl, potassium chloride (KCl), MgSO4, KH2PO4, NaHCO3, CaCl2 and d-glucose (The North Medical Chemical Reagent Factory, Taijin, China); thapsigargin, ATP, norepinephrine (NE), acetylcholine (ACh), Nω-nitro- l-arginine methylester (l-NAME), ethylenebis(oxyethylenenitriilo)tetraacetic acid (EGTA) and dimethyl sulfoxide (DMSO) (Sigma Chemical Co., St. Louis, MO, U.S.A.); Wogonin (Chinese Institute for Drug and Biological Product Control, Beijing, China). DMSO was used as a solvent for wogonin. Distilled water was used to dissolve all other drugs. All concentrations are described as the final concentration of the medium in the organ bath.

Animals and Ethics Statement Male Wistar rats weighing 250–300 g were used for the present study. Use of animals in this study was approved by the Tianjin University of Traditional Chinese Medicine Animal Care and Use Committee. The rats were group-housed in cages under controlled conditions of light, humidity and temperature. Water was provided ad libitum and standard particle feed were provided daily. All efforts were made to minimize the suffering of the animals and maximize their welfare.

Preparation of the Isolated Aorta The rats were sacrificed by decapitation. The thoracic aorta was rapidly removed and dissected from the rat. The aorta was cleaned of connective tissue and cut into 3–4 mm ring segments. Each ring was suspended in organ bath between two parallel stainless steel muscles. After 20 min incubation with wogonin, concentration–response curves to CaCl2 (1, 3, 10, 30 mM) were obtained in the presence and absence of wogonin (10 μM or 100 μM) for 20 min. Aortic rings were first allowed to equilibrate at 2.0 g tension in Ca2+-free Krebs’ solution, and then the rings were bled in Ca2+-free high-K+ (100 mM) Krebs’ solution, which was prepared by replacing an equimolar concentration of NaCl with KCl. After 20 min incubation with wogonin, concentration–response curves to CaCl2 were constructed. In vehicle control experiments, DMSO was added in the same volume as that used in the experiments with wogonin. These experiments were also performed in endothelium-denuded aorta.

Measurement of Ca2+ Influx and Intracellular Ca2+ Release Primary vascular smooth muscle cells (VSMCs) were prepared from the thoracic aorta of 2–3-month old male Wistar rats via the tissue explants method, as described previously.22) The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C in humidified atmosphere of 95% air and 5% CO2. More than 98% of the cells were positive for smooth muscle-specific α-actin, and exhibited the typical “hill and valley” growth pattern. Confluent cell at passages 3 to 7 were used for experiments. VSMCs were plated in 96-well black-walled clear-base plates at a density of 2×104 cells per well in DMEM with 10% FBS for 24 h. After pretreatment with various concentrations of wogonin in the presence or absence of extracellular Ca2+, the cells were incubated with Calcium 6 reagent (Molecular Devices, Sunnyvale, CA, U.S.A.) for 2 h. Then the VSMCs were checked on a FlexStation III (Molecular Devices), to monitor fluorescence (ex=485 nm, em=525 nm) before and after treatment with KCl (60 mM) or ATP (100 μM). Intracellular Ca2+ mobilization was measured as relative fluorescence units (RFU) and expressed as percentage of RFU at 0s.

Statistics Statistical analyses were performed using SPSS 11.5. Relaxant responses are given as percentage relaxation relative to precontraction levels to NE or KCl. Data were shown as mean±standard error of the mean (S.E.M.) from n number of experiments. Statistical significance was estimated by independent samples t-test between two groups. A p-value of less than 0.05 was regarded to be significant.

RESULTS

Effects of Wogonin on Rat Aortic Rings To investigate

rat plasma after intragastric administration of wogonin (5 mg/kg).14) So the route of administration of wogonin might be intravenous administration based on the clinical administration method.15)
the vasodilatory effect of wogonin, NE (1 µM) or KCl (60 mM) was used to contract the endothelium-intact aortic rings. Wogonin (0.1–100 µM) induced vasorelaxation in endothelium-intact aortic rings precontracted with NE (1 µM, n=10, Fig. 2A) or KCl (60 mM, n=3, Fig. 2B). Besides, NE (0.01, 0.1, 1 µM) or KCl (7.5, 15, 30, 60 mM) was used to contract the aortic rings in the presence and absence of wogonin (10 or 100 µM) for 20 min. NE (0.1, 1, 10 µM, n=6) or KCl (7.5, 15, 30, 60 mM, n=8) induced concentration-dependent contractions of rat aortic rings in Krebs’ solution. Pretreatment with 10 mM and 100 µM wogonin reduced the potency of contractile responses to NE (n=6, Fig. 2C) or KCl (n=8, Fig. 2D).

Effects of L-NAME and Endothelial Denudation on Vasorelaxation to Wogonin

Because wogonin induced vasorelaxation in endothelium-intact aortic rings precontracted with NE (1 µM) or KCl (60 mM), we investigated the involvement of endothelium in vasorelaxation to wogonin. Wogonin (0.1–100 µM) induced vasorelaxation in endothelium-intact aortic rings precontracted with NE (1 µM, n=10). Relaxant responses to wogonin were not inhibited by L-NAME (100 µM, n=8) and endothelial denudation (n=12) (Fig. 3).

Effects of Wogonin on Initial Fast and Sustained Phases Induced by NE

The contractile response of aortic ring to NE can be separated into initial and sustained phases. Because pretreatment with 10 and 100 µM wogonin could reduce the potency of contractile responses to NE (0.1, 1, 10 µM) and the vasodilatory effect of wogonin is not dependent on endothelial cells, we investigated the effects of wogonin on Initial Fast and Sustained Phases induced by NE in endothelium-denuded aortic rings. The initial contraction was first initi-
ated with NE (1 µM) in Ca²⁺-free Krebs’ solution (containing 1 mM EGTA) and the sustained contraction was induced by further addition of CaCl₂ (10 mM) in rat endothelium-denuded aortic rings. Data are shown as mean±S.E.M. *p<0.05, **p<0.01, as compared with the control.

Effects of Wogonin on CaCl₂ Induced Concentration-Dependent Contractions in Ca²⁺-Free High-K⁺ Krebs’ Solution in Rat Aortic Rings  The present study demonstrated that wogonin could inhibit KCl-induced vasoconstriction. Because high K⁺ induced contraction by increasing extracellular Ca²⁺ influx via voltage-gated Ca²⁺ channels, we investigated whether wogonin could inhibit extracellular Ca²⁺ influx in Ca²⁺-free high-K⁺ Krebs’ solution. CaCl₂ (1, 3, 10, 30 mM) induced concentration-dependent contractions of endothelium-denuded aortic rings in calcium-free buffer depolarized by 100 mM KCl. Pretreatment with wogonin (10, 100 µM) significantly reduced the potency of contractile responses to CaCl₂ (n=7, Fig. 5).

Effects of Wogonin on Ca²⁺ Influx and Intracellular Ca²⁺ Release in Vascular Smooth Muscle Cells  In endothelium-intact aortic rings, wogonin not only blocked Ca²⁺ influx-dependent vasoconstriction by either NE or KCl, but also inhibited NE-induced tonic contraction, which depends on intracellular Ca²⁺ release. In order to further study the mechanisms, we investigated whether wogonin affect Ca²⁺ influx and intracellular Ca²⁺ release in primary VSMCs of rat. In the presence of extracellular Ca²⁺, VSMCs were treated with 1 µM thapsigargin to exhaust calcium store in sarcoplasmic and endoplasmic reticula. The increase in VSMCs [Ca²⁺]i evoked by 60 mM KCl was significantly suppressed by pretreatment of wogonin (10, 100 µM) (n=6, Fig. 6A). While in the calcium-free cell culture medium (containing 0.5 mM EGTA), wogonin exerted inhibitory effects on intracellular-calcium dependent [Ca²⁺]i increase induced by 100 µM ATP in a dose-dependent
manner (n=6, Fig. 6B). These results indicated that both extracellular-calcium dependent Ca\(^{2+}\) influx and intracellular Ca\(^{2+}\) release could be inhibited by wogonin treatment.

**DISCUSSION**

The present study investigated the vasodilatory effect of wogonin and its possible mechanisms. Wogonin (0.1–100 \(\mu M\)) caused concentration-dependent relaxations in endothelium-intact aortic rings precontracted with NE (1 \(\mu M\)) or KCl (60 mM). Preincubation with wogonin (10, 100 \(\mu M\)) for 20 min significantly inhibited the contractile responses to NE (0.1, 1, 10 \(\mu M\)) or KCl (7.5, 15, 30, 60 mM). Relaxant responses to wogonin were not inhibited by l-NAME (100 \(\mu M\)) and endothelial denudation. In a Ca\(^{2+}\)-free Krebs’ solution, wogonin did not only block Ca\(^{2+}\) influx-dependent vasoconstriction by either NE (1 \(\mu M\)) or KCl (100 mM), but also inhibited NE (1 \(\mu M\))-induced tonic contraction, which depends on intracellular Ca\(^{2+}\) release. Besides, wogonin suppressed the elevation of [Ca\(^{2+}\)]i, induced by KCl (60 mM) after exhausting calcium store in sarcoplasmic and endoplasmic reticula with thapsigargin (1 \(\mu M\)) or by ATP (100 \(\mu M\)) in VSMCs.

Vascular endothelium, which releases endothelium-dependent vasodilators, plays an important role in maintaining normal function of vascular tension. NO is a main endothelium-dependent vasodilator, formed by NO synthase using L-arginine as a substrate. NO penetrates vascular smooth muscle cells and activates soluble guanylyl cyclase which catalyzes guanosine triphosphate to transform into cGMP. cGMP-activated protein kinase G reduces sensitivity of contractile elements. The vasodilatory effect of wogonin may be related to its ability to interfere with both extracellular Ca\(^{2+}\) influx and Ca\(^{2+}\) in endoplasmic reticulum release.

In conclusion, it is clearly shown that wogonin induces endothelium-independent vasorelaxation. The vasodilatory effect of wogonin may be related to its ability to interfere with both extracellular Ca\(^{2+}\) influx and Ca\(^{2+}\) in endoplasmic reticulum release.

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**Conflict of Interest** The authors declare no conflict of interest.

**REFERENCES**

2. Kwan CY, Chen CX, Deyama T, Nishibe S. Endothelium-dependent vasorelaxant effects of the aqueous extracts of the *Eucommia ulmoides* Ooliv. bark, is an endothelium- and NO-independent vasodilatory flavonoid. These results are important for validating the traditional use of *Eucommia ulmoides* Ooliv. bark and developing novel antihypertensive agents. However, the potential signal pathway is still unclear. Our previous study found that wogonin exhibited phytoestrogen activities, which shows extensive cardiovascular bioactivity.28 We need to do more work to study whether the potential signal pathway of wogonin-induced responses is associated with its phytoestrogen activities.

In conclusion, it is clearly shown that wogonin induces endothelium-independent vasorelaxation. The vasodilatory effect of wogonin may be related to its ability to interfere with both extracellular Ca\(^{2+}\) influx and Ca\(^{2+}\) in endoplasmic reticulum release.