Vasorelaxant effect of osthole on isolated thoracic aortic rings in rats

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Abstract
OBJECTIVE: To investigate the effect of osthole on isolated thoracic aortic rings, and to determine the potential mechanism of action.

METHODS: Thoracic aortas were isolated from Wistar rats, and were suspended in tissue organ chambers for vascular tension measurement. The effect of cumulative osthole (10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, and 10^{-1} mol/L) on endothelium-intact and endothelium-denuded thoracic aortic rings pre-contracted with phenylephrine (PE, 10^{-5} mol/L) or KCl (6 × 10^{-3} mol/L) was recorded. Histomorphological changes of thoracic aorta were analyzed by hematoxylin-eosin. The effects of different osthole concentrations on endothelium-intact aortic rings, which were pre-inhibited with the non-selective nitric oxide synthase inhibitor L-Arg(NO_{2})·OMe·HCl (3 × 10^{-4} mol/L), endothelium-derived nitric oxide synthase inhibitor Nω-nitro-L-arginine (3 × 10^{-4} mol/L), guanylate cyclase inhibitor 1H-[1,2,4] oxadiazolo [4,3-a] quinoxaline-1-one (10^{-5} mol/L), cyclooxygenase inhibitor indometacin (10^{-5} mol/L), and the Ca^{2+}- activated potassium channel inhibitor tetraethylammonium nitrate (10^{-4} mol/L), and then contracted with PE, were examined. Aortic rings incubated with osthole (10^{-5} mol/L), phenolamine (10^{-5} mol/L), or verapamil (10^{-5} mol/L) in Ca^{2+}-free Krebs-Henseleit solution (KHS) were stimulated with PE or KCl.

RESULTS: There was a dose-dependent increase in vasorelaxation of isolated thoracic aortic rings (both with and without endothelium) with increasing osthole concentration. Hematoxylin-eosin staining showed that osthole significantly improved thoracic aorta ring morphology. Compared with the control group, there were also significant differences after incubation with L-Arg(NO_{2})·OMe·HCl, Nω-nitro-L-arginine, and 1H-[1,2,4] oxadiazolo [4,3-a] quinoxaline-1-one (P < 0.05 for all). The relaxation rate of the rings in the osthole group incubated with indometacin and tetraethylammonium nitrate were similar to controls. In Ca^{2+}-free KHS, the PE-induced contraction was similar between the osthole (4.37% ± 0.41%) and control (4.21% ± 1.33%) groups. However, after cumulative CaCl_{2} (0.5, 1, 1.5, 2, 2.5, and 3 mmol/L), the Ca^{2+}-induced contraction was significantly inhibited in the osthole and phenolamine groups compared with controls (P < 0.05). After cumulative CaCl_{2}, was added to Ca^{2+}-free KHS (high K^{+} concentration), the contraction rate was significantly higher than both of the control and the osthole groups (P < 0.05). The contraction rate in the osthole group was higher than the verapamil group (P < 0.05).
CONCLUSION: Osthole has a vasorelaxant effect on isolated rat thoracic aortic rings, via inhibition of both receptor-operated and voltage-dependent Ca^{2+} channels in arterial smooth muscle, leading to decreased Ca^{2+} influx, and via inhibition of nitric oxide release on arterial endothelial cells.

Keywords: Aorta; thoracic; Osthole; Phentolamine; Verapamil; Vasorelaxation; Calcium; Nitric oxide

INTRODUCTION

Duhuo (Radix Angelicae Bisserratae) is a well-known pungent Traditional Chinese Medicine, and has been used for thousands of years as a remedy for arthritis, lumbago, edema, headache, thrombosis, and blood status. According to the theory of Traditional Chinese Medicine, Duhuo (Radix Angelicae Bisserratae) can promote the flow of Qi and activate blood circulation, predominantly through its vasorelaxant effect. We previously reported that an intestinal absorption solution of Duhuo (Radix Angelicae Bisserratae) had a significant relaxation effect on isolated thoracic aorta from rats. Osthole (7-methoxy-8-coumarin) is one of the most effective and bioactive chemical constituents extracted from Duhuo (Radix Angelicae Bisserratae), and was reported to show strong anti-arrhythmia and anti-tumor actions, and to protect the brain from neuronal apoptosis and ischemia-reperfusion injury. Intravenous injection of osthole to anesthetized dogs was also reported to reduce blood pressure, while Ogawa et al. reported that 3 weeks of 0.05% osthole feeding significantly reduced systolic blood pressure of male spontaneously hypertensive rats. Thus, in the present study, we examine the effects of osthole on thoracic aortic contraction, and the potential mechanisms of action.

MATERIALS AND METHODS

Experimental animals

Thirty-six specific pathogen-free male Wistar rats (280-300 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., China (Certification No. SCXK [Beijing] 2012-0001). All rats received free access to food and water. The study started when the rats were reared in the animal observation room after 3 days acclimatization. All experimental procedures were according to the Regulations for the Administration of Affairs Concerning Experimental Animals of People’s Republic of China.

Drug preparation

Osthole (≥ 98%, lot No. 141228) was purchased from Chengdu Best-reagent Co., China. Osthole was prepared in dimethyl sulfoxide (≥ 99.9%, lot No. SHBC3313V; Sigma-Aldrich, Co., Missouri, USA) and stored at −30°C until use.

Instruments

The following experimental instruments were used in this study: multichannel physiologic recorder (MP150; BIOPAC Systems, Inc., California, USA); tissue organ bath system (Radnoti LLC, USA); constant temperature water bath (Thermo Fisher Scientific, Massachusetts, USA); magnetic stirrer (IKA, Staufen, Germany); electronic balance (SQP; Sartorius Scientific Instrument Co. Ltd., Beijing, China); pipettes (Research plus; Eppendorf China Ltd., Shanghai, China); tissue embedded box (Citotest Labware Manufacturing Co., Ltd., Jiangsu, China); automatic tissue hydroextractor (Excelsior; Thermo Scientific, Cheshire, UK); tissue embedding machine (Histostar; Thermo Scientific); Shandon Finesse Me+ (Thermo Scientific); digital section bath (Thermo Scientific); slimline hotplate (Thermo Scientific); and a biological microscope (DM4000, Leica Microsystems Ltd., Germany).

Reagents

Phenylephrine (PE; ≥ 98%, lot No. K5QH-JB) and acetylycholine chloride (Ach; ≥ 98%, lot No. 46GDM-C) were purchased from Tokyo Chemical Industry Development Co., Ltd. (Tokyo, Japan). Phentolamine was purchased from Sigma-Aldrich, Co. Verapamil hydrochloride (lot No. L75939) was purchased from Fluorochem Ltd. (Derbyshire, UK). L-Arg (NO)·Ome · HCl (L-NAME; ≥ 98%, lot No. LE30Q205), Nω-Ac-L-arginine (L-NNA; ≥ 98%, lot No. L550Q10), indomethacin (Indo; ≥ 98%, lot No. LF80Q13), and tetraethylammonium nitrate (TEA; ≥ 98%, lot No. LT80Q34) were purchased from J & K Scientific Ltd. (Beijing, China). 1H-[1,2,4] oxadiazolo [4,3-α] quinoxaline-1-one (ODQ; lot No. A5465437) was purchased from APOLLO Scientific Ltd. (Stockport, Britain). All other reagents were analytical grade.

Nutrient solution preparation

The composition of the Krebs-Henseleit solution (KHS) was 118.96 mmol/L NaCl, 4.73 mmol/L KCl, 25.0 mmol/L NaHCO₃, 2.54 mmol/L CaCl₂, 1.17 mmol/L MgSO₄·7H₂O, 1.17 mmol/L KH₂PO₄, and 11.1 mmol/L glucose. The Ca²⁺-free KHS had no CaCl₂, but contained 0.05 mmol/L EGTA. The solutions were prepared and stored at 4°C before use.

Preparation of isolated thoracic aortic rings

Each rat was anesthetized with intraperitoneal injection of 10% chloral hydrate (4 mL/kg body weight), and the thoracic aorta was immediately removed and immersed in ice-cold KHS. The aorta was cleaned of all perivascular connective tissue and cut into three rings (4-5 mm in length). The rings were placed into tissue organ chambers filled with 5 mL KHS at 37.4°C that was continuously infused with 95% O₂ and 5%
CO₂, and were mounted on a force transducer connected to the multichannel physiological recorder. After stabilization for 30 min, the resting tension (1 g) was applied for 1 h, during which time the KH solution was replaced every 15 min. After stabilization, KCl (6 × 10⁻² mol/L) was used to contract the rings. The rings were then rinsed after reaching the smooth contraction amplitude. If necessary, this step was repeated several times to induce the maximum contraction amplitude. If the contraction amplitude was higher than 1 g, then we proceeded to the next step or switched to a new aortic ring.

To examine the direct effect of osthole on smooth muscle, the rings were denuded of endothelium by gently rubbing the internal surface with a toothpick. Successful removal of the endothelium was demonstrated by Ach testing. The rings were stimulated with PE (10⁻⁴ mol/L). After reaching the maximum contraction amplitude, the rings were relaxed by a concentration gradient of Ach (10⁻⁵, 10⁻⁶, 10⁻⁷, 10⁻⁸, and 10⁻⁹ mol/L), and the relaxation rate was calculated. If the relaxation rate was > 70%, the ring was regarded as endothelium-intact. The removal of the endothelium was considered successful if the relaxation rate was < 10%. The rings were rinsed dozens of times and equilibrated for at least 30 min before the next step.

**Effect of osthole on thoracic aortic rings pre-contracted with KCl or PE**

The aortic rings, with or without endothelium, were pre-contracted with KCl (6 × 10⁻² mol/L) or PE (10⁻⁴ mol/L). After reaching the maximum contraction amplitude, the rings were relaxed by a concentration gradient of osthole (10⁻⁵, 10⁻⁶, 10⁻⁷, 10⁻⁸, and 10⁻⁹ mol/L) and 10⁻³ mol/L KHS was added. The rings were then incubated with osthole (5 × 10⁻⁵ mol/L). All analysis was performed by Student’s t-test and plotted with GraphPad Prism v5.0 (GraphPad Software, Inc., USA). Values of \( P < 0.05 \) were considered statistically significant.

**Effect of osthole on thoracic aortic rings pre-contracted with KCl**

Compared with the control group, the KCl-contracted aortic rings were significantly relaxed by osthole in both the endothelium-intact group and the endothelium-denuded group. There was a significant difference in relaxation with osthole treatment between the endothelium-intact group and the endothelium-denuded group. The former was relaxed more than the latter. In addition, the relaxation rate increased with accumulated concentration of osthole (Figures 1, 2).

**Effect of osthole on thoracic aortic rings pre-contracted with PE**

The vasodilatation with osthole treatment was dose-dependent. Compared with the control group, the PE-contracted aortic rings were significantly relaxed by osthole in both the endothelium-intact group and the endothelium-denuded group. There was a significant difference in relaxation with osthole treatment between the endothelium-intact group and the endothelium-denuded group (Figures 3, 4).

**Effect of osthole on histomorphology of thoracic aortic rings in rats**

In the blank group, the inner membrane was smooth, and the endothelial cells were intact without local defects. The middle membrane showed no signs of proliferation, and the smooth muscle cells were long and spindle shaped, and neatly arranged. In the KCl + control group and the PE + control group, the surface of the endometrium was not smooth. The endothelial...
cells protruded and had partly detached. The middle membrane was thickened and the smooth muscle cells were disordered. The intimal surface of the KCl + osthole group and the PE + osthole group was not smooth, and the smooth muscle cells were arranged neatly, which was obviously improved compared with the control group. Thus, osthole significantly improved the morphology of the thoracic aorta rings that were pre-stimulated with PE and KCI (Figure 5).

Effect of osthole on thoracic aortic rings incubated with blockers

Both of the relaxation rates in the L-NAME group and the L-NNA group were much slower, which significantly different from that in the control group. There was also a significant difference in the relaxation rate between the ODQ group and the control group. The relaxation rate was higher than that in the control group. The relaxation rates in the Indo group and the TEA group were similar to that in the control group. (Figures 6, 7).

Effect of incubation of osthole on thoracic aortic rings in Ca²⁺-free KHS

In Ca²⁺-free KHS, the contraction rate of the PE + osthole group (4.37% ± 0.41%) was similar to that in the PE + control group (4.21% ± 1.33%). After cumulative CaCl₂ (0.5, 1, 1.5, 2, 2.5, and 3 mmol/L) was added, the contraction rate induced by CaCl₂ in the PE + control group was significantly greater than that in the PE + osthole group. In the meanwhile, the PE + osthole group was much higher than that in the PE + phenolamine group, but similar to that in the PE + verapamil group. In addition, after cumulative CaCl₂ was added to the Ca²⁺-free KHS (with high K⁺ concentration), the rings in the KCl + control group contracted much more strongly than that in the KCl + osthole group. The contraction rate in the KCl + osthole group was similar to that in the KCl + phenolamine group, but higher than that in the KCl + verapamil group (Figures 8, 9).
In the present study, we found that osthole produced a dose-dependent relaxing effect on PE-contracted and KCl-contracted thoracic aortic rings. The relaxation rate in the endothelium-intact group was higher than in the endothelium-denuded group.

**DISCUSSION**

In the present study, we found that osthole produced a...
Graph traces show changes in the tension in the endothelium-intact thoracic aortic rings in response to PE ($10^{-6}$ mol/L) and osthole ($10^{-9}$, $10^{-8}$, $10^{-7}$, $10^{-6}$, and $10^{-5}$ mol/L), which were separately incubated by the following blockers. L-NAME group incubated by L-NAME ($3 \times 10^{-4}$ mol/L) for 15 min; L-NNA group incubated by L-NNA ($3 \times 10^{-4}$ mol/L) for 15 min; Indo group incubated by Indo ($10^{-5}$ mol/L) for 15 min; ODQ group incubated by ODQ ($10^{-5}$ mol/L) for 15 min; TEA group incubated by TEA ($10^{-5}$ mol/L) for 15 min. A: control group; B: L-NAME group; C: L-NNA group; D: ODQ group; E: Indo group; F: TEA group; PE: phenylephrine; L-NAME: L-Arg (NO$_2$)-Ome·HCl; L-NNA: Nω-Nitro-L-arginine; ODQ: 1H-[1,2,4] oxadiazolo[4,3-a] quinoxaline-1-one; Indo: indo-methacin; TEA: tetraethylammonium nitrate.
that in the endothelium-denuded group, indicating that the vasorelaxant effect of osthole was partly endothelium-dependent.

Endothelial cells can synthesize and secrete a variety of vasoactive substances, which act on vascular smooth muscle cells. For example, endothelial cells can secrete vasodilator factors such as nitric oxide (NO), prostaglandin I\(_2\) (PGI\(_2\)), and endothelium-derived hyperpolarizing factors.\(^\text{10,11}\) NO is the product of L-arginine catalyzed by NO synthase (NOS). NO diffuses into smooth muscle cells to activate guanylate cyclase, which increases the concentration of intracellular cyclic guanosine monophosphate (cGMP) and decreases the concentration of free Ca\(^{2+}\), thus causing vasodilation.\(^\text{10,11}\)

In the present study, compared with the control group, pretreatment with either the non-selective NOS inhibitor L-NAME or the endothelium-derived NOS inhibitor L-NNA significantly reduced the PE-induced contraction of aortic rings, indicating that the vasorelaxant effect was endothelium-dependent and related to release of NO. The guanylate cyclase inhibitor ODQ also inhibited the relaxation effect of osthole, providing further support that osthole relaxes aortic rings through the NO-cGMP pathway.

Arachidonic acid produces PGI\(_2\) under the action of cyclooxygenase (COX) and prostacyclin synthase, PGI\(_2\) can also activate adenylate cyclase to produce cyclic adenosine monophosphate, which induces vasodilatation. Indo is a COX inhibitor, which results in reduced PGI\(_2\) synthesis, thus inhibiting vasodilation.\(^\text{16,17}\) In the present study, there were no changes in vasorelaxation when osthole was applied to aortic rings pre-incubated with Indo, suggesting that PGI\(_2\) may not be involved in the vasodilator action of osthole.

Endothelium-derived hyperpolarizing factors are a class of substances that can hyperpolarize vascular smooth muscle cells by acting on relevant ion channels (e.g., K\(^+\) channels), causing vasodilation.\(^\text{18}\) There are four types of K\(^+\) channels expressed on blood vessels (voltage-dependent K\(^+\) channels, inward rectifier K\(^+\) channels, calcium-activated K\(^+\) channels [K\(_{\text{Ca}}\)], and Adenosine Triphosphate-sensitive K\(^+\) channels). Under normal physiological conditions, K\(_{\text{Ca}}\) channels have the highest density.\(^\text{17,18}\) To examine for a role of K\(_{\text{Ca}}\) channels on vasorelaxation in the present study, we pretreated the rings with TEA (K\(_{\text{Ca}}\) channel inhibitor), and found that relaxation was similar to that in the control groups. Thus, K\(_{\text{Ca}}\) channels were not involved in the relaxation with osthole treatment.

Ca\(^{2+}\) is a key factor involved in vascular smooth muscle contraction, which primarily involves extracellular Ca\(^{2+}\) influx and intracellular Ca\(^{2+}\) release. Extracellular Ca\(^{2+}\) influx mainly occurs via Ca\(^{2+}\) channels on the cell membrane, which are predominantly voltage-dependent Ca\(^{2+}\) channels (VDCC\(_{\text{L}}\)) and receptor-operated Ca\(^{2+}\) channels (ROCC\(_{\text{L}}\)).\(^\text{19,20}\) In the present study, osthole had a significant relaxing action on both KCl-contracted and PE-contracted aortic rings. PE is an \(\alpha\)-adrenergic...
Figure 8 Experimental process diagram of incubation of osthole on thoracic aortic rings in Ca\[^{2+}\]-free KHS
Graph traces show changes in the tension in the endothelium-denuded thoracic aortic rings in Ca\[^{2+}\]-free KHS in response to CaCl\(_2\) (0.5, 1, 1.5, 2, 2.5, 3 mmol/L), which were separately incubated by osthole (10\(^{-5}\) mol/L), phentolamine (10\(^{-5}\) mol/L) or verapamil (10\(^{-5}\) mol/L) for 10 min. PE + osthole group and KCl + osthole group incubated with osthole (10\(^{-5}\) mol/L); PE + phentolamine group and KCl + phentolamine group incubated with phentolamine (10\(^{-5}\) mol/L); PE + verapamil group and KCl + verapamil group incubated with verapamil (10\(^{-5}\) mol/L). A: PE + control group; B: PE + osthole group; C: PE + phentolamine group; D: PE + verapamil group; E: KCl + control group; F: KCl + osthole group; G: KCl + phentolamine group; H: KCl + verapamil group. PE: phenylephrine; Ach: acetylcholine chloride; KHS: Krebs-Henseleit solution.
In the present study, when we add KCl to the CaCl₂ and CaCl₂-free KHS, the Ca²⁺ influx and reduced intracellular Ca²⁺. In the present study, when we used Ca²⁺-free KHS, the contraction induced by PE was caused only by release of intracellular Ca²⁺. Compared with the control group, pretreatment with osthole did not reduce the PE-induced contraction, demonstrating that osthole did not inhibit Ca²⁺ release. When we added cumulative CaCl₂, pretreatment with osthole and phenotamine (an ROCC inhibitor) significantly reduced the Ca²⁺-induced contraction compared with the control group, suggesting that osthole can reduce Ca²⁺-influx by inhibiting ROCCs. KCl-induced contraction is caused by a high extracellular K⁺ concentration and cell membrane depolarization, leading to VDCC opening and Ca²⁺ influx. In the present study, when we added the cumulative CaCl₂ to the Ca²⁺-free KHS (with high K⁺ concentration), the Ca²⁺-induced contraction in the control group was significantly higher than that in the osthole group; this effect was similar to that observed with VDCC inhibitor (verapamil) pretreatment. Thus, osthole can inhibit the Ca²⁺ release influx through VDCC.

In conclusion, osthole has a dose-dependent vasorelaxant effect on isolated rat thoracic aortic rings. There are two possible mechanisms of action of vasodilatation. One is via the endothelium-dependent NO-cGMP pathway, affecting NO release onto endothelial cells. The other is via inhibition of ROCCs and VDCC on vascular smooth muscle, which leads to decreased Ca²⁺ influx and reduced intracellular Ca²⁺.

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