Research paper

Mechanisms of the dilator action of the Erigerontis Herba on rat aorta

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Ethnopharmacological relevance: Erigerontis Herba is widely used as a traditional Chinese medicine and is commonly used for neuroprotection and vascular protection.

Aim of study: In this study, the vasodilator effects of Erigerontis Herba (DZXX) were investigated using rat isolated aorta rings.

Material and method: The involvement of endothelium in the vasorelaxation was studied by comparing response of endothelium-intact and endothelium-denuded aorta rings which precontracted with U46619. The involvement of K⁺ channels was studied by pretreatment of the aorta rings with various K⁺ channel inhibitors. The involvement of Ca²⁺ channel was studied by incubating aorta rings with Ca²⁺-free solution, primed with U46619 prior to eliciting contraction by addition of Ca²⁺ solution.

Results: DZXX (0.2–2 mg/ml) induced a concentration-dependent relaxation on U44619-precontracted aorta rings with EC₅₀ of 0.354 ± 0.036 mg/ml. Removal of endothelium or pretreatment with a BKCa inhibitor iberiotoxin, KIR inhibitor barium chloride or a non-selective K⁺ channel inhibitor 4-aminopyridine produced no effect on the DZXX-induced vasorelaxation. However, pretreatment with a KATP inhibitor glibenclamide or a non-selective K⁺ channel inhibitor tetraethylammonium produced significant inhibition on the DZXX-induced vasorelaxation by 29.9% and 21.3%, respectively. Pretreatment with DZXX (0.4, 1.2 and 2 mg/ml) produced a concentration-dependent inhibition on Ca²⁺-induced vasconstriction.

Conclusions: These results suggest that the vasodilator effect of DZXX was endothelium-independent, mediated by decreasing the influx of Ca²⁺ by calcium channel inhibition and increasing the influx of K⁺ by opening of a KATP channel.

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1. Introduction

Traditional Chinese medicine (TCMs) has been used clinically with a long history in China and can be regarded as potential sources for drug discovery. Erigerontis Herba (Pinyin: Dengzhanxixin), a whole flowering plant of Erigeron breviscapus (Vant.) Hand.-Mazz. (Compositae), is a well-known folk medicinal herb that mainly grows in Yunnan province of Southwest China, has been widely used as a folk remedy by the native people for treatment of cardiovascular diseases and cerebrovascular diseases such as coronary heart disease, angina pectoris and paralysis (Zhu et al., 2009). Erigerontis Herba preparations have been developed in various forms and one of most widely used preparation is the Erigerontis Herba injection (EHI) as indicated in Pharmacopoeia of the People’s Republic of China (Han et al., 2012). EHI showed remarkable effects in the treatment syndromes caused by ischemic stroke and coronary heart disease such as blood stasis and stagnation, apoplectic hemiplegia, extremity numbness, eye–mouth deviation, speech dysphasia, and chest stuffiness and pain (Liao et al., 2010). Erigerontis Herba is rich in flavonoids (Zhang et al., 2007) which have been proven to possess neuroprotective effects against reactive oxygen species, the aggregation of β-amyloid, and the induction of cell death by β-amyloid (Zhu et al., 2007). One of its major phenolic compound is scutellarin that has been proven to have neuroprotective and cardioprotective effects against cerebral ischemic injury-induced apoptosis in PC-12 cells (Zhang et al., 2009) and rat model of cerebral ischemia...
and myocardial ischemia induced by middle cerebral artery occlusion (MCAO) and ligation-induced acute myocardial infarction (MI) (Lin et al., 2007; Zhang et al., 2009). Other phenolic compounds includes 3,5-dicafeoyl quinic acid, 1,5-dicafeoyl quinic acid and Erigoster B, and 4,5-dicafeoyl quinic acid (Yue et al., 2000) was found to possess anti-oxidative effect, anti-platelet aggregation activity, and anti-embolic effect (Sun and Zhao, 2009). However, experimental investigation on the vasodilator effect of Erigeronitis Herba is rare. Vasodilators are used to treat hypertension, heart failure, angina and stroke, etc. We hypothesized that the use of Erigeronis Herba would be beneficial to both cardiovascular and cerebrovascular patients by its vasodilator effect. Therefore, we designed the present study to investigate the vasorelaxant activity and mechanism of action of the ethanol extract of the Erigeronitis Herba (DZXX). For this purpose, isolated rat thoracic aorta rings were used to assess the vasorelaxant activities of DZXX.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals were purchased from Sigma, USA included iberitoxin, barium chloride (BaCl2), 4-aminopyridine (4-AP), glibenclamide and tetraethylammonium (TEA). All reagents were of analytical purity.

2.2. Herbal preparation

Erigeronis Herba (Dengzhanxixin) was provided by Kumming Institute of Botany (KIB) from Yunnan province. Small quantity of the herb was deposited as voucher specimens in the museum of the Institute of Chinese Medicine, the Chinese University of Hong Kong, with voucher specimen numbers of 2010–3283. Briefly, 250 g of the Erigeronis Herba was allowed to soak in 10-fold of absolute ethanol (2.5 L) for 1 h followed by reflux for another 1 h. The extract was collected and the extraction was repeated for another 1 h with 2.5 L absolute ethanol. The extracts were filtered and the extracts were collected and concentrated under reduced pressure at 40 °C. The concentrated extract was then lyophilized to dry ethanol extract of Erigeronitis (DZXX) for further use.

2.3. Identification of chemical markers in DZXX

Stock solutions of (1) Scutellarin, (2) 3,5-dicafeoyl quinic acid, (3) 1,5-dicafeoyl quinic acid, (4) Erigoster B, and (5) 4,5-dicafeoyl quinic acid were prepared individually in HPLC-grade methanol to produce a final concentration of 1 mg/ml. The stock solution was filtered through a syringe filter of 0.2 μm. The 5 stock solutions were mixed and further diluted with methanol to give a series of final concentration of 1 mg/ml. The stock solution was injected into UPLC for analysis.

DZXX samples were prepared by dissolving the dried ethanol extract in HPLC-grade methanol to give a final concentration of 1 mg/ml, and was filtered through a syringe filter of 0.2 μm. Sample solution of 5 μl was injected into the UPLC system.

Analysis was performed on a Waters ACQUITY UPLC system (Waters, Milford, MA, USA). The analytical method was developed using a Water ACQUITY UPLC BEH C-18 column (2.1 × 150 mm², 1.7 μm) (Waters, Milford, MA, USA). The mobile phase consisted of 0.1% formic acid in acetonitrile (solvent A) and 0.1% formic acid in water (solvent B), with an isocratic elution program: 15% A and 85% B. The flow rate was set to 0.3 ml/min, injection volume was 5 μl, column temperature was maintained at room temperature (23 °C) using a column oven, and detection was carried out using a PDA detector at λmax 330 nm.

2.4. Animal and preparation of rat aorta ring

Male Sprague-Dawley rats (240–260 g) were used and provided by the Laboratory Animal Services Centre (LASEC) of the Chinese University of Hong Kong. All animal procedures were conducted under license from the Government of the Hong Kong Special Administrative Region (HKSAR) and approval by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong (Approval number: 09/065/MIS). Rats were anesthetized by exposure to diethyl ether. The thoracic aorta was removed and immersed in Krebs–Henseleit solution (in mM): NaCl (118); KCl (4.7); CaCl2 (1.25); MgCl2 6H2O (1); NaHCO3 (25); and glucose (13.5) at pH 7.4. Aorta was cut into 3 mm rings and mounted between stainless-steel hooks on Grass isometric transducers (Grass Instruments, Quincy, USA) for isometric tension recording in organ chambers filled with Krebs–Henseleit solution. The aorta was gassed with 95% O2/5% CO2 to maintain pH7.4. The tension was transformed into digital signal which was displayed on a computer connected to a PowerLabs (AD Instruments, Sydney, Australia) for data acquisition (sampling rate 100/s).

2.5. Experimental protocols

The same experimental protocols as described in our recent article were adopted (Ng et al., 2011). First, the organ bath was filled with Krebs–Henseleit solution at 37 °C, and bubbled with 95% O2/5% CO2. Rings were stretched to 1.5 g of baseline tension and equilibrated for 30 min. In experiments with endothelium-intact aorta, endothelium integrity was confirmed by the observation of more than 70% relaxation induced by 0.3 μM acetylcholine (Ach) after pre-contract by 0.3 μM phenylephrine (Phe). Wash-out was carried out by draining and replacement of buffer solution from a syringe. Rings were then pre-contracted with thromboxane A2 mimetic, U46619 (15 nM) to establish stable contractile tone. Subsequently, a concentration-response curve of DZXX was obtained by cumulative addition to the aortic rings and the tension was monitored for 2 h. To test for involvement of endothelium-dependent mechanisms, one of the each paired aorta rings obtained was subjected to mechanical removal of the endothelium by rubbing of the vessel lumen with a steel wire 30 min prior to precontraction by 15 nM U46619. Cumulative concentration-response curve for DZXX was then constructed and compared with that obtained in the paired artery with intact endothelium.

To investigate the involvement of potassium channels in endothelium-independent mechanisms, a cumulative concentration–response curve was obtained on the endothelium-denuded artery ring following the above protocol. The artery ring was then washed three times at an interval of 15 min, and then subjected to pretreatment with one of the following potassium channel inhibitors 20 min prior to precontraction by 15 nM U46619: a large-conductance Ca2+–activated K+ channels (BKCa) inhibitor ibero- toxin (100 nM), an inwardly rectifying K+ channels (Kir) inhibitor barium chloride (30 μM), a voltage-activated K+ channels (Kv) inhibitor 4-aminopyridine (1 mM), an ATP-sensitive K+ channels (KATP) inhibitor glibenclamide (10 μM) and a non-selective K+ channel inhibitor tetraethylammonium (10 mM). A second cumulative concentration–response curve was then constructed and compared with the first curve obtained in the absence of inhibitors.

To investigate the involvement of calcium channels in the relaxation response, aorta rings were equilibrated in Ca2+-free solution.
Krebs–Henseleit solution containing 0.2 mM EGTA for 30 min and washed three times at 10 min interval. U46619 (15 nM) was added as a primer to activate thromboxane receptors. CaCl$_2$ in concentration of 0.01–4.44 mM was then added at 5 min intervals to produce cumulative concentration–response curve. When maximum vasoconstriction was achieved by addition of CaCl$_2$ solution, the ring was washed and equilibrated for 30 min, and then incubated for 10 min with one of the following: 0.4, 1.2, 2.0 mg/ml DZXX. Concentration–response curves to cumulative addition of CaCl$_2$ were then repeated and compared with control curves obtained in the absence of DZXX.

2.6. Statistical analysis

Data were expressed as mean ± S.E.M. Differences between curves were analyzed by two-way ANOVA using Graphpad Prism software. All tests were two-tailed and the significance was set at $P=0.05$.

Fig. 1. Representative HPLC chromatograms of (A) a mixture of spiked standards and (B) an ethanol extract of Herba Erigonertis (DengZhanXiXin) measured at wavelength of 330 nm. (1) Scutellarin, (2) 3,5-dicaffeoyl quinic acid, (3) 1,5-dicaffeoyl quinic acid, (4) Ergoster B, and (5) 4,5-dicaffeoyl quinic acid.
3. Results

3.1. Chemical constituents in DZXX

The retention time of the five chemical constituents in Erigerontis Herba, namely Scutellarin, 3,5-dicaffeoyl quinic acid, 1,5-dicaffeoyl quinic acid, Erigoster B, and 4,5-dicaffeoyl quinic acid were 9.45, 15.3, 15.9, 17.2 and 27.1 min, respectively (Fig. 1). All five chemical constituents in DZXX were identified in their corresponding retention times.

3.2. Effect on U46619-precontracted tone

The precontractile tone produced by 15 nM U46619 was around 1.5 g. Cumulative addition of vehicle produced no effect on the precontractile tone. A concentration–response curve for DZXX (0.2–2 mg/ml) was established with rat aorta rings with or without endothelium. DZXX induced a concentration-dependent relaxation on U44619-precontracted aorta rings with intact endothelium with EC50 of 0.354 ± 0.036 mg/ml and a maximal vasorelaxation of 111.3% (Fig. 2), which is beyond the magnitude of the U46619-precontractile tone. To test the involvement of endothelium in the DZXX-induced vasorelaxation, the experiment was repeated in endothelium-denuded aorta rings; vasorelaxation induced by DZXX was not altered by the removal of endothelium (P > 0.05, Fig. 2).

3.3. Effect of K⁺ channel blockers on DZXX-induced vasorelaxation in endothelium-denuded aorta rings

K⁺ channel blockers were used to test the involvement of K⁺ channels in endothelium-denuded aorta rings. As shown in Fig. 3, pretreatment with the BKCa inhibitor iberiotoxin (Fig. 3A), KIR inhibitor barium chloride (Fig. 3B) or Kv inhibitor 4-aminopyridine (Fig. 3C) produced no effect on the DZXX-induced vasorelaxation (P=0.908, P=0.985 and P=0.107, respectively). As shown in Fig. 4, pretreatment with the KATP inhibitor glibenclamide (Fig. 4A) or a non-selective potassium channel inhibitor tetraethylammonium (Fig. 4B) produced significant inhibition on the DZXX-induced vasorelaxation by 29.9% and 21.3%, respectively (P < 0.0001 for both).
followed by bonferroni post-hoc test. **P<0.01, ***P<0.001 as compared with the same DZXX treatment without inhibitor.

3.4. Effect on Ca2+-induced contraction

Ca2+-free Krebs–Henseleit solution was used in this study. Without Ca2+ ion, priming the aorta ring with U46619 had no effect on the resting vascular tone. With cumulative addition of Ca2+ (CaCl2 solution, 0.01–4.44 mM) produced a stepwise increase in the vascular tone (Fig. 5). After addition of Ca2+ up to 4.44 mM, a maximal contraction was induced to 0.344±0.020 g. After incubation with 0.4, 1.2, 2 mg/ml DZXX, the maximal contraction induced by Ca2+ was significantly reduced to 0.314±0.027 g (8.7% reduction; P<0.0001), 0.188±0.030 g (45.3% reduction; P<0.0001), 0.058±0.013 g (83.1% reduction; P<0.0001). While incubating with 35 ng/ml nifedipine, the maximal contraction induced by Ca2+ was significantly reduced to 0.032±0.003 g (90.8% reduction; P<0.0001).

4. Discussion

The present study describes the vasodilator effects of the ethanol extract of Erigerontis Herba (DZXX) in rat aorta ring on the contraction induced by U46619, a thromboxane A2 agonist and its related mechanism. It first identified the vasorelaxant effect of DZXX, which concentration-dependently relaxed both endothelium-intact and endothelium-denuded aorta rings. These results indicated that the vasorelaxation action of DZXX could be endothelium-independent. Pretreatment with the KATP inhibitor glibenclamide and a non-selective potassium channel inhibitor tetraethylammonium produced no effect on the DZXX-induced vasorelaxation. Pretreatment with the KATP inhibitor glibenclamide or a non-selective potassium channel inhibitor tetroxyammonium produced significant inhibition on the DZXX-induced vasorelaxation suggested that the involvement of KATP channel in this vasodilator action. The involvement of calcium channel was also detected and DZXX could inhibit the influx of Ca2+ influx into smooth muscle cells, and produced its vasodilator action.

Erigeron Herba contains a total amount of caffeoyl conjugates (chlorogenic acid) around 4% which included, 3,5-dicaffeoyl quinic acid, 1,5-dicaffeoyl quinic acid, Erigoster B, and 4,5-dicaffeoyl quinic acid. It also contains total flavonoids less than 1%, e.g. scutellarin. The concentration of the total caffeoyl conjugates in much higher than that of the total flavonoids in this plant (Yue et al., 2000).

Scutellarin, is a flavonoid, has been extensively used to treat cardiovascular diseases such as hypertension, angina pectoris,
myocardial infarction and stroke in China for many years. It was found that scutellarin caused a concentration-dependent relaxation in both endothelium-intact and endothelium-denuded rat aorta rings (Pan et al., 2008). However, Pan et al. showed that the vasorelaxation of scutellarin was not inhibited by glibenclamide and TEA which indicated that the vasodilator action was not related to K_{ATP} channel. Our results showed that DZXX-induced vasodilation through K_{ATP} channel might not be due to the presence of scutellarin but other chemical compounds in DZXX. In addition, scutellarin alleviated Ca^{2+}-induced vasoconstriction in Ca^{2+}-depleted pretreated rings and this may be related to the action of DZXX. In a study of animal model of hypertension, they demonstrated that scutellarin is protective against chronic hypertension-induced activation of brain TLR4 and subsequent inflammatory responses. They showed that scutellarin possesses anti-inflammatory and anti-apoptotic properties and lowers blood pressure (Chen et al., 2013). Chronic hypertension causes cardiac hypertrophy, characterized by low-grade inflammation and accompanied by increased expression and activity of TLR4, and elevated gene expression of TNF-α and IL-6 in cardiac tissue (Eissler et al., 2011, Wang et al., 2013). This may imply that DZXX may have beneficial effect for chronic heart diseases.

Chlorogenic acid (CGA), an ester of caffeic acid and (-)-quinic acid, is in abundant amount in Erigeron Herba. CGAs are potent antioxidants (Clifford, 1999, Olthof et al., 2001, Stalmach et al., 2010) and can be found in many foods and drinks, most notably in coffee (Olthof et al., 2001). Other dietary sources of CGA include tea, cocoa, pears, berry fruits, citrus fruits, tomatoes and eggplants (Clifford, 1999). The physiological actions of CGA have carcinostatic activity, glucose/lipid metabolism–improving activity, antioxidant activity on low-density lipoprotein peroxidation and anti-hypertensive effect in human (Laranjinha et al., 1994, Morishita et al., 1997, Rodriguez de Sotillo and Hadley, 2002, Kozuma et al., 2005). In recent years, both basic and clinical studies have revealed that chlorogenic acid can have an anti-hypertension effect (Zhao et al., 2012). Mechanistically, the metabolites of CGAs attenuate oxidative stress (reactive oxygen species), which leads to the benefit of blood-pressure reduction (Suzuki et al., 2002, Kozuma et al., 2005) through improved endothelial function and nitric oxide bioavailability in the arterial vasculature (Ochiai et al., 2004, Suzuki et al., 2006). However, our results showed that DZXX produced vasorelaxation through endothelium-independent pathway solely. Why chlorogenic acid in DZXX cannot produce endothelium-dependent vasorelaxation? Will any compound in DZXX could interact with and diminish or abolish its endothelium-dependent vasorelaxation? Suzuki et al. reported that, in coffee, hydroxyhydroquinone could interfere with the chlorogenic acid-induced restoration of endothelial function in spontaneously hypertensive rats (Suzuki et al., 2008). Further experiment to study the individual chlorogenic acid will be needed to elucidate the differentiation.

In conclusion, our study demonstrated that DZXX which contain both scutellarin and different forms of chlorogenic acids would induce endothelium-independent and concentration-dependent relaxation of rat aorta rings through inhibition of Ca^{2+} influx in the vascular smooth muscle cells and opening of K_{ATP} channels. The present findings suggest that DZXX could potentially improve blood circulation and hypertension.

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