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Mechanisms of the cerebral vasodilator actions of isoflavonoids of Gegen on rat isolated basilar artery

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ABSTRACT

Ethnopharmacological relevance: Gegen (root of *Pueraria lobata*) is used in traditional Chinese medicine for treatment of cardiovascular diseases. In this study, the relaxant actions of three of its isoflavonoids; puerarin, daidzein, and daidzin, were investigated on rat-isolated cerebral basilar artery.
Materials and methods: Rat basilar artery rings were precontracted with 100 nM U46619. Involvement of endothelium-dependent mechanisms was investigated by mechanical removal of the endothelium and inhibitors of nitric oxide synthase (NOS) and cyclooxygenase (COX) enzymes. Adenylyl cyclase- and guanylyl cyclase-dependent pathways were investigated using their respective inhibitors 9-(tetrahydro-2-furanyl)-9H-purine-6-amine (SQ22536) and 1H-[1,2,4]oxadiazolo [4,3-[alpha]]-quinoxalin-1-one (ODQ). K^+ channels were investigated by pretreatment of the artery rings with various K^+ channel inhibitors, and Ca^{2+} channels were investigated in artery rings incubated with Ca^{2+}-free buffer and primed with 100 nM U46619 for 5 min prior to adding $CaCl_2$ to elicit contraction.
Results: Puerarin, daidzein, and daidzin produced concentration-dependent relaxation of the artery rings with concentration that produced 50% inhibition (IC_{50}) of 304 ± 49 μM, 20 ± 7 μM, and 140 ± 21 μM, respectively. Removal of the endothelium produced no change on their vasorelaxant responses except the maximum response (I_{max}) to puerarin was inhibited by 28%. The NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; 100 μM) also produced 45% inhibition on the puerarin-induced vasorelaxant response, but not the COX inhibitor flurbiprofen (10 μM). SQ22536 (100 μM) and ODQ (100 μM) did not affect the vasodilator responses to puerarin, daidzein and daidzin, but glibenclamide (1 μM), tetraethylammonium (TEA, 100 mM) or a combination of K^+ channel inhibitors (100 nM iberiotoxin + 1 mM 4-aminopyridine + 100 μM barium chloride + 1 μM glibenclamide + 100 mM TEA) reduced their I_{max}. The contractile response to $CaCl_2$ was attenuated by 61% and 34% in the presence of daidzein and daidzin, respectively, whereas, puerarin did not significantly affect the contraction.
Conclusions: The vasorelaxant action of daidzein and daidzin involved opening of K^+ channels and inhibition of Ca^{2+} influx in the vascular smooth muscle cells. There is no evidence supporting involvement of endothelium-derived relaxing factors (EDRFs) in their actions. In contrast, puerarin produced vasodilatation via an endothelium-dependent mechanism involving nitric oxide production and an endothelium-independent pathway mediated by the opening of K^+ channels. The cerebral vasodilator activities of all these three isoflavonoids may be beneficial to patients with obstructive cerebrovascular diseases.

1. Introduction

Gegen is the dried root of *Pueraria lobata*. This plant is native to South East Asia and it has been utilized as a food source, fodder and medicine for thousands of years (Luo et al., 2007; Csurhes, 2008). This herbal medicine has been ascribed with a plethora of biological activities including anti-pathogenic, anti-hypertensive, anti-platelet, anti-inflammation, anti-apoptotic, and anti-diabetic activities (Song et al., 2007; K.H. Wong et al., 2011). It is often used alone or in combination with other herbs for improving liver function, enhancing detoxification processes, regulating cardiac functions and aiding weight loss (K.H. Wong et al., 2011). Gegen is a rich source of polyphenolic compounds, including isoflavones, isoflavonoid glycosides, coumarins, puerarols and their

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derivatives. Isoflavonoids are its major bioactive constituents, and these include puerarin, daidzin, daidzein, 8-C-apiosyl-glucoside, genistin and genistein, of which, puerarin, daidzin and daidzein have been found to possess cardiovascular tonic effects (Choo et al., 2002; Hintz and Ren, 2004; Yan et al., 2006).

Puerarin, 7-hydroxy-3-(4-hydroxyphenyl)-1-benzopyran-4-one 8-β-D-glucopyranoside, is the most abundant active ingredient in Gegen. Various pharmacopoeias and companies commonly employ puerarin as the gold standard of quality of Gegen medicinal materials. Since the isolation of puerarin from Gegen in the late 1950s, its comprehensive biological actions have been well-documented (K.H. Wong et al., 2011). Currently, there are three main dosage forms of puerarin in clinical application, including injection, capsule and tablet. The injection form is used widely in China for the treatment of coronary heart disease, angina pectoris, cardiac infarction, obstruction of retinal artery and vein, sudden deafness, cerebrovascular diseases, viral myocarditis and diabetes (K.H. Wong et al., 2011). The beneficial effects of puerarin in treatment of these conditions may be related to its ability to reduce lactate dehydrogenase (LDH) release, prevent cell death, depolarize mitochondrial membrane, inhibition of reactive oxygen species (ROS) release, promote angiogenesis and inhibit calcium influx (Guo et al., 2004; Gao et al., 2006, 2007; Zhang et al., 2006).

Daidzein and daidzin can be found in soy bean as well as Gegen (Verkasalo et al., 2001; Kim et al., 2010). Daidzein is an aglycone of puerarin. It is a phytoestrogen used for treatment of menopausal-related disorders such as menopausal symptoms, osteoporosis, breast cancer, and cardiovascular diseases (Verkasalo et al., 2001; Patisaul and Jefferson, 2010). Daidzein was demonstrated to have beneficial effects on cerebral ischaemia and alcohol toxicity in animal studies (Nakagawa et al., 2006; Zhang et al., 2010). Daidzin is also known as daidzein 7-O-glucoside. It is reported to have diverse biological activities, such as protection on hepatic failure, anti-alcohol addiction, anti-oxidation and preventing the development of osteonecrosis (Toda and Shirataki, 2001; Li et al., 2005; Lowe et al., 2008; Kim et al., 2009). Both daidzin and daidzein have been reported to improve brain functions (Kouki et al., 2003; Kim et al., 2010).

The cerebral basilar artery is one of the arteries that supplies the brain with oxygen-rich blood. Ischaemic stroke due to obstruction of the basilar artery carries a poor prognosis as it causes hypoxia and ischaemia of the brainstem that could lead to paralysis of all extremities, heavy disturbances in sensation, difficulty in swallowing and difficulty in respiration. Recent evidence indicates a decoction of Danshen (*Salvia miltiorrhiza*) and Gegen could be beneficial in treatment of obstruction vascular diseases as it was shown to have cardio-protective and cerebral vasodilator actions (Lam et al., 2010; S.M. Wong et al., 2011). The water soluble constituents of Danshen, salvianolic acid B and danshensu, probably contribute to these actions of the decoction (Lam et al., 2006b, 2007, 2010); the other contributing agents are probably the isoflavonoid constituents in Gegen (Lam et al., 2010). In the present study, we have isolated and quantified three isoflavonoids; puerarin, daidzein and daidzin, in a Gegen aqueous extract. Furthermore, to confirm a role for these isoflavonoids in protecting the cerebral circulation, we have investigated their relaxant properties on rat isolated cerebral basilar arteries and compared their mechanisms of actions.

2. Materials and methods

2.1. Herbal preparation

Raw Gegen herb was purchased from local herbal shop in Sichuan province. Manufacturing of the herbal extract was carried out by National Engineering Research Center for Modernization of traditional Chinese medicines (TCM), a Good Manufacturing Practice (GMP) compliance manufacturer in Mainland China. Briefly, the raw material of Gegen was washed, cut, dried, and soaked with 10 fold of water (v/w) for 1.5 h followed by extraction at 100 °C for 1 h. Two subsequent extractions were carried out by adding 8 fold of water (v/w) and extracted for 1 h and 0.5 h. The extracts were collected and combined and then concentrated at 65 °C under reduced pressure of −0.07 MPa to give dry powder (batch#: PL05092008-H_2O). A voucher specimen of the present Gegen herb (voucher specimen#: 2008-3166b) is kept in the museum of Institute of Chinese Medicine, The Chinese University of Hong Kong.

2.2. Identification and quantification of puerarin, daidzein and daidzin in Gegen water extracts

Identification and quantification of puerarin, daidzein and daidzin in Gegen water extract were conducted according to our previous study with minor modifications on the instrument and chromatographic conditions (Chang et al., 2008). Briefly, the liquid chromatographic system used was a Waters HPLC system (Waters, Milford, MA, USA) equipped with 2695 solvent delivery module and a 996 photodiode (PDA) UV detector. The chromatographic separation of the analytes was achieved by using an Agilent Eclipse XDB-C18 column (250 mm × 4.6 mm i.d.; 5 μm particle size) connected to an Agilent C18 guard column. The auto-sampler was set at 4 ± 5 °C. The mobile phase consisting of 0.5% acetic acid in acetonitrile (solvent A) and 0.5% acetic acid in water (solvent B) was run with gradient elution at a flow rate of 1 ml/min. The linear gradient elution was carried out according to the following program: solvent A was kept in 5% for the first 5 min, and then increased to 10%, 17%, 35% and 90% in the next 13 min, 12 min, 10 min and 30 min, respectively, then returned to 5% in 5 min and equilibrated for 15 min before the next injection.

2.3. Animals

Experiments were performed on male Sprague–Dawley rats (250–300 g) bred and kept by the Laboratory Animal Services Center of the Chinese University of Hong Kong. All experiments were performed under license from the Government of the Hong Kong SAR and endorsed by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong.

2.4. Isolation and mounting of blood vessels

Rats were killed by gassing with carbon dioxide. The basilar artery was carefully isolated from the brain. Fat and connective tissue were removed under a dissecting microscope. Four vessel rings of 2 mm length were cut from the artery and then mounted with 25 μm steel wires to separate 5 ml tissue baths of an integrated myograph system (Danish Myo Technology Model 619M) for tension recording. The tissue baths were filled with Krebs–Henseleit solution (gassed with 95% O_2/5% CO_2; pH 7.4; 37 ± 1 °C) of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.18, $NaHCO_3$ 25, glucose 10. Tension signals were relayed to a MacLab 4 amplifier and saved to a Macintosh PowerMac computer system (sampling rate 100 s^{-1}).

2.5. Experimental protocols

The same experimental protocols as described in our recent article were adopted with minor modifications (Lam et al., 2010). During the initial 45 min equilibration period, the vessel rings were stretched until the resting tension held steady at approximately 0.6 g. The preparations were then checked for contractile response