Effects of total flavones from *Dendranthema morifolium* on vasocontraction and proliferation of vascular smooth muscle cells

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Abstract. Pharmacological studies have shown that the active components in *Dendranthema morifolium* exhibit protective effects against ischemia/reperfusion injury; however, its pharmacological action on blood vessels has not yet been investigated. The purpose of the present study was to assess the effects of the total flavones extracted from *D. morifolium* (Ramat.) Tzvel. cv. Hangju (FDM) on the vasocontraction and proliferation of vascular smooth muscle cells (VSMCs). The tension of rat thoracic aortic rings was measured using a mechanical force transducer attached to a recording system. FDM induced a dose-dependent relaxation of rings with endothelium pre-contracted by either phenylephrine (PE; 10^{-6} mol/l) or a high concentration of potassium chloride (KCl; 60 mmol/l). FDM did not significantly affect the vasorelaxant effects on mechanically removed endothelium. In endothelium-denuded aortic rings depolarized by 60 mmol/l KCl, FDM inhibited the contraction induced by Ca^{2+} . FDM reduced the transient contraction caused by PE in a Ca^{2+} -free solution, but did not affect the contraction induced by phorbol ester. Furthermore, FDM inhibited the proliferation of VSMCs with or without growth stimulation by insulin. In conclusion, that the vasorelaxation induced by FDM in rat aortic rings is not dependent on the endothelium but is mediated via a reduction of the influx of extracellular Ca^{2+} through the voltage-dependent and receptor-operated channels and via the inhibition of the release of intracellular Ca^{2+} in VSMCs. The anti-proliferative activity of FDM suggests that it may be beneficial in inhibiting atherosclerosis.

Introduction

Dendranthema morifolium (Ramat.) Tzvel. cv. Hangju of the genus *Chrysanthemum* and the Asteraceae family is

widely used in south China, either as a fragrant floral tea or as an anti-inflammatory herb in Traditional Chinese Medicine. According to component analysis, *D. morifolium* contains various nutrients, vitamins A and B, and flavonoids (1,2). The main flavonoid components include luteolin-7-*O*- β -D-glucoside, apigenin-7-*O*- β -D-glucoside, acacetin-7-*O*- β -D-glucoside and anthocyanins (1,3). It has been reported that these components have a hypotensive effect in rats under normal conditions (4) and increase the contractility and the coronary flow in isolated and re-perfused rat hearts, thereby protecting the heart against ischemia/reperfusion injury (5,6). However, to the best of our knowledge, the possible pharmacological effects of *D. morifolium* components on vessels and the underlying mechanism of action have not been studied. The present study hypothesized that total flavones extracted from *D. morifolium* (Ramat.) Tzvel. cv. Hangju (FDM) may have a direct relaxant effect on vasculature. The vasorelaxant effects of FDM and the potential underlying mechanisms of action were therefore investigated.

As a cluster of cardiovascular risk factors, the metabolic syndrome is increasingly being recognized as an important factor in the pathophysiological mechanisms underlying atherosclerosis and is a target of therapies (7). The metabolic syndrome is also termed syndrome of insulin resistance. Insulin can stimulate the over-proliferation of vascular smooth muscle cells (VSMCs) through the mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol-3 kinase (PI3K)-Akt pathway (8). The over-proliferation of VSMCs is one of the key factors in atherosclerosis and the restenosis following percutaneous transcatheter angioplasty (9,10). It has been reported that the flavones and flavonoids extracted from other plants inhibit the proliferation of VSMCs and leiomyoma cells (11,12). Therefore, the present study examined the effects of FDM on the proliferation of VSMCs.

Materials and methods

Drugs and chemicals. Acetylcholine (ACh), phenylephrine (PE) and phorbol 12,13-diacetate were purchased from Sigma-Aldrich (St. Louis, MO, USA). FDM was prepared by extraction (with 70% ethanol) of the dried flowers of *D. morifolium* (Ramat.) Tzvel. cv. Hangju, which were obtained from Tongxiang City (China), as described in a previous study (13). The FDM was identified by Professor Huidi Jiang (Department

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Preparation of rat aortic rings. Male Sprague Dawley (SD) rats (weight, 240–300 g; age, 6–8 weeks) were purchased from the Animal Center, Zhejiang Academy of Medical Sciences (Hangzhou, China). All procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals and the experimental protocol of the present study was approved by the ethics committee of Zhejiang University. Rats were anesthetized with chloral hydrate (0.4 g/kg intraperitoneally (i.p.); Shenggong Biotech Co., Shanghai, China) and sacrificed by decapitation. Thoracic aortas were immediately harvested and maintained in a Krebs solution with the following composition: 118 mmol/l NaCl, 25 mmol/l NaHCO₃, 1.2 mmol/l KH₂PO₄, 1.2 mmol/l MgSO₄, 4.7 mmol/l KCl, 1.25 mmol/l CaCl₂ and 10 mmol/l glucose (Shenggong Biotech Co.) at 4°C. Aortas were cleared of adipose and connective tissue and then cut into 3 mm-wide rings. A forceps was inserted into the lumen of the aortic ring and the endothelium was gently removed. Rings were suspended from an iron hook connected with a force transducer in chambers containing 10 ml Krebs solution at 37°C and with streaming of 95% O₂ and 5% CO₂ gas. The preparations were passively stretched to an optimal tension of 2 g after 15 min of equilibration with no tension. The equilibration time was 60 min and the Krebs solution was refreshed every 15 min. Isometric tension was recorded by transducers connected to a recording system (MacLab, ADInstruments, Bella Vista, Australia) (14). The function of the rings was first tested in the presence of KCl (60 mmol/l; Shenggong Biotech Co.) at least three times until a reproducible and stable contractile response was obtained. The function of the endothelium was tested using ACh (10⁻⁵ mol/l; Shenggong Biotech Co.) to induce at least 70% relaxation pre-contraction with PE (10⁻⁶ mol/l).

Culture of VSMCs and cell proliferation assay. According to the protocol of a previous study (15), chloral hydrate (0.4 g/kg, i.p.) was used to anesthetize the male SD rats (240–300 g) prior to sacrifice by decapitation. Thoracic aortas were rapidly removed under aseptic conditions and stored in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and antibiotics (100 g/ml streptomycin and 100 U/ml penicillin; Sigma-Aldrich). The aortas were dissected, longitudinally opened and cut into 1 x 1-mm pieces following removal of the endothelium and adventitia. Cell cultures were maintained under 5% CO₂ at 37°C in an incubator and the medium was replaced twice a week. VSMCs were identified by a typical 'hill and valley' growth pattern and confirmed by immunofluorescence staining for α -smooth muscle actin (Sigma-Aldrich), which is identified by green fluorescence. Confluent cells at passage 3–7 were used in all experiments.

The effects of FDM on VSMC proliferation were assayed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method with the MTT Cell Proliferation and Cytotoxicity Assay kit (Beyotime Institute of Biotechnology, Haimen, China) as previously described (16). The VSMCs

were transferred onto a 96-well plate. After 20 h of culture, 15 μ l MTT (5 mg/ml) was added to each well and the culture medium was replaced with dimethyl sulfoxide (DMSO; Sigma-Aldrich) after 4 h. The absorbance of every well was measured at a wavelength of 570 nm using a spectrophotometer (Multiskan MK3; Thermo Fisher Scientific, Inc.).

Assessment of the effects of FDM on PE- and KCl-induced vascular contraction. After a steady contraction of the aortic rings with or without endothelium induced by PE (10⁻⁶ mol/l) or KCl (60 mmol/l) was achieved, FDM (0.005, 0.015, 0.05, 0.15 or 0.5 g/l), dissolved in DMSO, was cumulatively added to the Krebs solution, and the results were used to generate a concentration-response curve.

Assessment of the effects of extracellular and intracellular calcium on FDM-induced reduction of vasocontraction. In the first series of experiments, aortic rings were first incubated in Ca²⁺-free Krebs solution containing 50 mol/l ethylene glycol bis(aminoethylether)-tetraacetic acid (EGTA; Shenggong Biotech Co.) and 60 mmol/l KCl for 20 min. FDM at 5x10⁻² g/l was then added to the Krebs solution. In order to obtain a concentration-response curve, Ca²⁺ (0.25–5 mmol/l) was then cumulatively added after 10 min.

In the second series of experiments, after endothelium-denuded rings were incubated in Ca²⁺-free Krebs solution containing 50 μ mol/l EGTA for 15 min, PE (10⁻⁶ mol/l) was used to induce the first transient contraction. In order to allow for the restoration of intracellular Ca²⁺ levels, Ca²⁺-free Krebs solution was then replaced with normal Krebs solution and the aortic rings were incubated in normal Krebs solution for at least 40 min. After the solution was rapidly replaced with Ca²⁺-free solution, the rings were incubated for another 15 min. In order to test the effects of FDM on the second contraction induced by 10⁻⁶ mol/l PE, 5x10⁻² g/l FDM was added to the solution after 15 min.

In the third series of experiments, 2x10⁻³ mol/l caffeine (Shenggong Biotech Co.) was used to elicit a transient contractile response in endothelium-scraped rings immersed in Ca²⁺-free Krebs solution. 5x10⁻² g/l FDM was added to the solution and the second contraction was induced again.

Assessment of the effects of FDM on phorbol ester-induced vasocontraction. In order to induce a stable contraction in endothelium-scraped rings, 10⁻⁶ mol/l phorbol ester (phorbol 12,13-diacetate; Shenggong Biotech Co.), an activator of protein kinase C, was added to Ca²⁺-free Krebs solution containing 50 μ mol/l EGTA, which the rings were immersed in. FDM was cumulatively added and the effects were recorded.

Assessment of the effects of FDM on the proliferation of cultured VSMCs. VSMCs at passages 3–7 and 70–90% confluence in 10-cm dishes were transferred to a 96-well plate. The growth of VSMCs was arrested by incubation with serum-free DMEM for 24 h prior to use.

In the first set of experiments, synchronized cells were treated in the presence or absence of FDM (0.0025, 0.01, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.28 and 2.56 g/l) for 24 h, and the effects of FDM on the cell proliferation were assessed using the MTT method as described above.

In the second set of experiments, cells were pre-incubated with insulin (100 nmol/l; Shenggong Biotech Co.) for 24 h. FDM (0.32 or 0.64 g/l) was added to the culture medium for another 24 h and the proliferation of the VSMCs was assessed using the MTT method.

Statistical analysis. Values are expressed as the mean $\pm$ standard deviation. An unpaired Student's t-test was used to compare differences between two groups. One-way analysis of variance and the Newman-Keuls test, followed by a post-hoc least-significant differences test were used to determine significant differences between multiple groups. SPSS 15.0 (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses. $P < 0.05$ was considered to indicate a statistically significant difference. GraphPad Prism software version 5.0 (GraphPad Software Inc., San Diego, CA, USA) was used for curve fitting.

Results

FDM exerts concentration-dependent vasorelaxant effects. After a steady contraction elicited by 10^{-6} mol/l PE was achieved, FDM was added to the aortic rings and was revealed to concentration-dependently induce vascular relaxation (Fig. 1). FDM at 0.5 g/l induced complete relaxation of aortic rings in endothelium-intact as well as endothelium-denuded aortic rings (Fig. 1A-C). Furthermore, FDM caused a concentration-dependent decrease of vasoconstriction induced by KCl (60 mmol/l), in endothelium-intact as well as -denuded aortic rings (Fig. 1D).

FDM reduces vasoconstriction induced by extracellular and intracellular calcium. After a steady contraction of endothelium-scraped aortic rings was induced by KCl (60 mmol/l) in Ca^{2+} -free Krebs solution, Ca^{2+} was cumulatively added to the solution, which was shown to concentration-dependently enhance vasoconstriction. The Ca^{2+} -induced vasoconstriction of the aortic rings was significantly inhibited by incubation with 5×10^{-2} g/l FDM for 10 min (Fig. 2A).

In another series of experiments, a contraction of endothelium-scraped aortic rings was transiently induced by 10^{-6} mol/l PE in Ca^{2+} -free Krebs solution. A second contraction was further induced by PE with or without 5×10^{-2} g/l FDM. As shown in Fig. 2, the ratio of the second to the first contraction was significantly reduced by pre-treatment with FDM for 10 min. Pre-treatment with FDM also reduced the ratio of the second to the first contraction elicited by 2×10^{-3} mol/l caffeine (Fig. 2B).

FDM does not affect phorbol ester-induced vasocontraction in a Ca^{2+} -free solution. As shown in Fig. 3, a sustained contraction of endothelium-scraped rings evoked by phorbol ester in Ca^{2+} -free Krebs solution with 50 $\mu\text{mol/l}$ EGTA was not effected by FDM.

FDM inhibits the proliferation of cultured VSMCs. FDM dose-dependently inhibited the proliferation of cultured VSMCs. It also reduced, but did not fully abrogate, insulin-induced increases in the proliferation of VSMCs (Fig. 4).

Figure 1. Concentration-dependent effects of FDM on contraction responses of endothelium-intact and endothelium-scraped aortic rings to (A-C) PE (10^{-6} mol/l) or (D) KCl (60 mmol/l). Values are expressed as the mean $\pm$ standard deviation ($n=8$ for +endothelium group, $n=9$ for -endothelium group and $n=10$ for the control group). * $P < 0.05$ and ** $P < 0.01$ vs. Control. FDM, total flavones from *Dendranthema morifolium* (Ramat) Tzvel. cv. Hangju; PE, phenylephrine.

Discussion

Although *D. morifolium* is widely used in southeast China, its pharmacological action on blood vessels has not been assessed, to the best of our knowledge. The present study revealed the

Figure 2. Effect of FDM (5 × 10⁻² g/l) on (A) the Ca²⁺-induced contraction of endothelium-scraped aortic rings depolarized with high K⁺ (60 mmol/l) and on (B) the ratio of the contractile responses to PE (10⁻⁶ mol/l) or caffeine (2 × 10⁻³ mol/l) in a Ca²⁺-free solution. Values are expressed as the mean ± standard deviation (n=5 in A and n=79 in B). *P<0.05, **P<0.01 vs. control. FDM, total flavones from *Dendranthema morifolium* (Ramat) Tzvel. cv. Hangju; PE, phenylephrine.

Figure 3. Effects of FDM on phorbol ester (10⁻⁶ mol/l)-induced contraction in endothelium-denuded aortas in Ca²⁺-free solution. Each data-point represents the result for one aorta and horizontal lines represent the mean values for the respective experimental groups (n=4). FDM, total flavones from *Dendranthema morifolium* (Ramat) Tzvel. cv. Hangju.

dose-dependent vasorelaxant effects of FDM in aortic rings from rats and investigated the potential underlying mechanisms. Furthermore, it was demonstrated that insulin-induced growth stimulation of VSMCs was inhibited by FDM.

The results of the present study revealed that FDM exerted a vascular relaxation effect and inhibited the contractions of aortic rings from rats evoked by either PE or KCl. The vasodilation induced by FDM in aortic rings was shown to be endothelium-independent. Therefore, the mechanism of action of FDM is unlikely to be mediated via any endogenous vasodilators, including prostacyclin and nitric oxide from endothelial cells. Ajay *et al* (17) demonstrated that the release of nitric oxide was partly involved in the vascular dilation effects of most of the flavonoids. In addition, Jiang *et al* (18) reported that the nitric oxide synthase (NOS) activity of aortic rings was significantly increased by pre-treatment with an ethyl acetate

Figure 4. Effects of FDM on the proliferation of cultured vascular smooth muscle cells. (A) Absorbency of formazan at 570 nm, which reflects cell proliferation. (B) Inhibition of VSMCs by FDM. (C) Effects of FDM on the proliferation of VSMCs stimulated by insulin (100 nmol/l). Values are expressed as the mean ± standard deviation (n=6). **P<0.01 vs. vehicle or control; *P<0.05, **P<0.01 vs. insulin; *P<0.05 vs. control. FDM, total flavones from *Dendranthema morifolium* (Ramat) Tzvel. cv. Hangju; VSMC, vascular smooth muscle cell.

extract of *D. morifolium*. Whether FDM causes any variation in NO levels and/or NOS activation requires further study.

The two main types of Ca²⁺ channel, voltage-dependent and receptor-operated channels, can be activated by KCl and PE, respectively. KCl causes vascular contraction mainly by inducing Ca²⁺ influx upon de-polarization of the cell membrane and activating voltage-dependent L-type Ca²⁺ channels (19).

In the present study, FDM inhibited KCl-induced vasoconstriction in endothelium-denuded rat aortas as well as Ca²⁺-induced contraction of aortic rings de-polarized by KCl, which demonstrated that FDM exerts its vascular dilation effect in part by inhibiting the voltage-dependent L-type Ca²⁺ channel. In addition, FDM significantly inhibited the PE-induced vascular contraction, indicating that it may also reduce influx of Ca²⁺ through receptor-operated Ca²⁺ channels.

PE, as a selective α -adrenoceptor agonist, induces vasoconstriction by opening receptor-operated Ca²⁺ channels.

More importantly, it evokes Ca^{2+} release from the sarcoplasmic reticulum by activating phospholipase C and the generation of diacylglycerol (DG) that activates myosin light chain kinase through protein kinase C (PKC) and inositol triphosphate (IP3) (20). Therefore, the present study investigated whether FDM exerts the vasorelaxant effects through inhibiting the intracellular mobilization of Ca^{2+} from the sarcoplasmic reticulum and diglyceride-dependent PKC-activated myosin light chain kinase in aortic rings. In order to demonstrate that the transient contraction response to PE was mainly due to IP3-induced Ca^{2+} release from the sarcoplasmic reticulum, several experiments of the present study were performed in a Ca^{2+} -free Krebs solution. FDM inhibited the PE-induced vascular contraction response in endothelium-scraped aortic rings in an extracellular Ca^{2+} -free environment, probably due to the inhibition of intracellular Ca^{2+} release. However, the vascular contraction response to phorbol ester was not affected by FDM in the concentration range that relaxed aortic rings induced to contract by pre-treatment with PE or KCl, suggesting that the relaxation response to FDM was not mediated through the PKC pathway.

Insulin can stimulate the overproliferation of VSMCs through the MAPK and the PI3K-Akt pathway (7). This over-proliferation is a key factor in atherosclerosis and the restenosis following stent implantation (8,9). The results of the present study showed that FDM inhibited the proliferation of VSMCs under normal conditions and also inhibited insulin-induced over-proliferation. Therefore, FDM has useful implications in the clinical treatment of diseases associated with the over-proliferation of VSMCs, including as atherosclerosis and restenosis after stent implantation. Further study is required to explore the underlying mechanisms of the inhibitory effects of FDM on the proliferation of VSMCs.

In conclusion, the present study indicated that FDM exerted a vasorelaxant effect on rat aortic rings in a concentration-dependent and endothelium-independent manner. The underlying mechanism is likely to include a reduction of the Ca^{2+} influx by FDM through voltage-dependent as well as receptor-operated channels to inhibit the release of intracellular Ca^{2+} in VSMCs. It was also demonstrated that FDM inhibited the proliferation of VSMCs under normal conditions and the over-proliferation induced by insulin.

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