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Original Article

Vasorelaxant and antihypertensive effects of formononetin through endothelium-dependent and -independent mechanisms

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Aim: To investigate the mechanisms underlying the vasorelaxant effect of formononetin, an *O*-methylated isoflavone, in isolated arteries, and its antihypertensive activity *in vivo*.
Methods: Arterial rings of superior mesenteric arteries, renal arteries, cerebral basilar arteries, coronary arteries and abdominal aortas were prepared from SD rats. Isometric tension of the arterial rings was recorded using a myograph system. Arterial pressure was measured using tail-cuff method in spontaneously hypertensive rats.
Results: Formononetin (1–300 μmol/L) elicited relaxation in arteries of the five regions that were pre-contracted by KCl (60 mmol/L), U46619 (1 μmol/L) or phenylephrine (10 μmol/L). The formononetin-induced relaxation was reduced by removal of endothelium or by pretreatment with *L*-NAME (100 μmol/L). Under conditions of endothelium denudation, formononetin (10, 30, and 100 μmol/L) inhibited the contraction induced by KCl and that induced by $CaCl_2$ in Ca^{2+}-free depolarized medium. In the absence of extracellular Ca^{2+}, formononetin (10, 30, and 100 μmol/L) depressed the constriction caused by phenylephrine (10 μmol/L), but did not inhibit the tonic contraction in response to the addition of $CaCl_2$ (2 mmol/L). The contraction caused by caffeine (30 mmol/L) was not inhibited by formononetin (100 μmol/L). Formononetin (10 and 100 μmol/L) reduced the change rate of Ca^{2+}-fluorescence intensity in response to KCl (50 mmol/L). In spontaneously hypertensive rats, formononetin (5, 10, and 20 mg/kg) slowly lowered the systolic, diastolic and mean arterial pressure.
Conclusion: Formononetin causes vasodilatation via two pathways: (1) endothelium-independent pathway, probably due to inhibition of voltage-dependent Ca^{2+} channels and intracellular Ca^{2+} release; and (2) endothelium-dependent pathway by releasing NO. Both the pathways may contribute to its antihypertensive effect.

Keywords: formononetin; arterial rings; vasodilatation; voltage-dependent Ca^{2+} channel; intracellular Ca^{2+} release; nitric oxide; spontaneously hypertensive rats; blood pressure

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Introduction

Hypertension is one of the most common cardiovascular diseases, which is a major risk factor for endothelial dysfunction, metabolic syndrome, diabetes, renal dysfunction, congestive heart failure, coronary artery diseases and stroke[1]. Clinically, various antihypertensive drugs such as diuretics, centrally acting adrenergic drugs, vasodilators, calcium channel blockers and angiotensin converting enzyme/receptor blockers have been used to treat hypertension. However, the efficacy of these drugs is only 40%–60%, and usually two or more antihypertensive drugs from different categories need to be combined to achieve optimal results. In addition, the side effects from these medications are an important concern[2].

To develop a safe and effective way for managing hypertension has long been a challenge for medical researchers and doctors. Many traditional Chinese medicinal herbs with vasorelaxant properties are conventionally used to treat hypertension[3]. Recently, interest in the use of medicinal herbs has risen exponentially, due to their low toxicity and wonderful therapeutical performance[4]. Therefore, we started a project to find a novel antihypertensive compound with vasorelaxant activity from traditional Chinese medicinal plants that are used as antihypertensive agents.

Phytoestrogens are plant substances found in many foods, which are structurally or functionally similar to estradiol. They have attracted much attention because of their potential beneficial role in prevention and treatment of cardiovascular diseases, bone metabolism disorders, breast cancers,

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and menopausal symptoms[5–9]. Isoflavones are a class of phytoestrogens naturally sourced from legumes such as soy beans and red clover[10]. Some isoflavones have been reported for treatment of hypertension, coronary heart diseases and cardiac infarct[11–14] through dilating arteries and increasing blood flow[15, 16]. Formononetin, an *O*-methylated isoflavone, is contained in the roots of *Astragalus membranaceus*[17, 18], liquorice[19], black cohosh[20, 21] and *Trifolium pratense* L[22]. Medicinal herbs containing formononetin have been used to treat cardiovascular diseases including hypertension in Asia for centuries[23]. Recently, Wu *et al* reported that formononetin has a vasorelaxant activity in isolated rat aorta rings[9]. However, the antihypertensive activity of formononetin and its vasorelaxant effect on arteries in different regions have not yet been studied. The aim of the present study was to determine whether formononetin is contributory to treating hypertension, and further to explore the potential mechanisms of its antihypertensive activity.

Materials and methods

Drugs and reagents

Formononetin was supplied by Department of Chemistry, Shaanxi Normal University, China. Phenylephrine, 9,11-dideoxy-11 alpha, 9 alpha-epoxymethano-prostaglandin F_2 alpha (U46619), 5-hydroxytryptamine (5-HT), noradrenaline, Triton X-100, propranolol, glibenclamide, tetraethylammonium, acetylcholine chloride (ACh), indomethacin, N^{ω}-nitro-*L*-arginine methyl ester (*L*-NAME), verapamil, lacidipine, sodium nitroprusside (SNP), caffeine, and dimethyl sulphoxide (DMSO) were purchased from Sigma Aldrich (St Louis, MO, USA). Fluo-3/AM was obtained from Biotium (Hayward, CA, USA). All other reagents were of analytical grade. Formononetin was dissolved in DMSO for *in vitro* use and dissolved in 10% Tween 80 solution for *in vivo* use. Fluo-3/AM and glibenclamide were dissolved in DMSO. Indomethacin was dissolved in ethanol. Other substances were dissolved in double distilled water. The concentrations are expressed as the final molar concentrations in the tissue baths.

Animals

Sprague-Dawley (SD) rats were obtained from Experimental Animal Center of Xi-an Jiaotong University College of Medicine, China. Male spontaneously hypertensive rats (SHRs) aged 20 weeks were obtained from Shanghai Slack Laboratory Animal Co Ltd, China. The study was approved by Ethics Committee of Xi-an Jiaotong University College of Medicine according to the principles outlined in the Declaration of Helsinki.

Arterial rings preparation

SD rats weighing 250–300 g were sacrificed by CO_2. The rat superior mesenteric arteries, renal arteries, cerebral basilar arteries, coronary arteries, and abdominal aortas were gently removed. The arteries were immersed in cold oxygenated Krebs solution containing the following composition (mmol/L): NaCl 119, $NaHCO_3$ 15, KCl 4.6, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $CaCl_2$ 1.5 and glucose 5.5. The arteries were dissected free of adhering tissue under a microscope. In the endothelium-denuded experiments, the endothelium was denuded by perfusion of the vessels for 10 s with 0.1% Triton X-100 followed by another 10 s with Krebs solution[24]. The vessels were then cut into 1 mm cylindrical segments.

Myograph experiments

The artery segments were threaded on two 40-μm-diameter stainless steel wires and mounted in Mulvany-Halpern myographs (Danish Myo Technology A/S, Aarhus, Denmark). One wire was connected to a force displacement transducer and attached to an analog-to-digital converter unit (AD Instruments, Hastings, UK). The other wire was attached to a movable displacement device, allowing fine adjustments of vascular tension by varying the distance between the wires. The data were recorded using Chart™ (AD Instruments, Hastings, UK). The mounted artery segments were immersed in temperature-controlled (37 °C) tissue baths containing 5 mL Krebs solution. The solution was continuously gassed with 5% CO_2 in O_2 resulting in a physiological pH at 7.4. The artery segments were equilibrated for 1.5 h before the experiments, and the cerebral artery and coronary artery segments were given a resting tension of 1.5 mN, while other segments a 3-mN tension. The contractile capacity of each vessel segment was tested by exposing the segment to a K^+-rich Krebs solution (with 60 mmol/L KCl) in which NaCl was exchanged for an equimolar concentration of KCl. Two reproducible high-K^+ contractions were obtained for standardization of the preparations.

The completeness of endothelium denudation was tested with ACh (10 μmol/L) after cerebral basilar arteries and coronary arteries were pre-contracted with 0.3 μmol/L 5-HT, and mesenteric arteries, renal arteries and abdominal aortas were pre-contracted with 10 μmol/L noradrenaline. No relaxation in response to ACh in the denuded preparation was regarded as functional removal of the endothelium. The endothelium was considered intact when such an ACh response caused more than 30% relaxation[25].

To determine the relaxant effect, the segments were pre-contracted, and once the sustained tension was obtained, formononetin (1–300 μmol/L) was added cumulatively to induce a concentration-dependent response. Some experiments were performed in both endothelium-intact and endothelium-free mesenteric arteries to determine whether the relaxant effect of formononetin was endothelium-dependent. Phenylephrine and formononetin were added after some mesenteric artery rings were pre-treated with *L*-NAME and/or indomethacin for 20 min so as to determine the involvement of nitric oxide (NO), prostaglandins (PGs) and endothelium-derived hyperpolarizing factor (EDHR) in the relaxant effect of formononetin. In order to evaluate the role of potassium (K^+) channels and β-adrenoceptors in the vasorelaxant effect of formononetin, some mesenteric artery rings were pre-treated with tetraethylammonium, $BaCl_2$, glibenclamide, or propranolol for 20 min before the addition of vasoconstrictor and formononetin.

To determine the involvement of voltage-dependent Ca^{2+} channels (VDCC), α_1-adrenoceptors and 5-HT receptors in the relaxant effect of formononetin, concentration-response curves of KCl, $CaCl_2$ in Ca^{2+}-free depolarized medium, phenylephrine and 5-HT in the presence of formononetin were constructed. Some mesenteric artery rings were incubated in a Ca^{2+}-free Krebs solution to determine whether the relaxant effect of formononetin was due to its inhibition of intracellular Ca^{2+} release or extracellular Ca^{2+} influx[26, 27].

The smooth muscle function was assessed at the end of each experiment. The mesenteric arteries, renal arteries and abdominal aortas were pre-constricted with phenylephrine (10 μmol/L), while the cerebral basilar arteries and coronary arteries were pre-contracted with 0.3 μmol/L 5-HT. Concentration-dependent vasorelaxation to the endothelium-independent vasodilator (SNP: 0.1 nmol/L–10 μmol/L) was tested. Only experiments on vessels with SNP-induced relaxation being more than 95% were accounted as valid[28].

Determination of tissue $[Ca^{2+}]_i$ in the mesenteric artery

SD rats weighing 120–130 g were sacrificed by CO_2, and the superior mesenteric arteries were gently removed. The arteries were immersed in cold HEPES-Krebs solution (pH 7.4) of the following composition (mmol/L): NaCl 135, KCl 5, $MgSO_4$ 1.2, $CaCl_2$ 2.5, glucose 10 and HEPES 8.4. Tissues adhering to the arteries were cleaned under a microscope. Each mesenteric artery was cut into ring segments with a length of about 3 mm and mounted on a U-shaped stainless steel wire. The artery rings with the wire were placed in the bottom of the chamber close to cover glass, followed by immediate immersion in HEPES-Krebs solution containing 10 μmol/L Fluo-3/AM, which was then added with DMSO, formononetin (10 μmol/L), formononetin (100 μmol/L) and verapamil (1 μmol/L), respectively. After 30 min, the artery rings were washed three times with HEPES-Krebs solution (pH 7.4) containing DMSO, formononetin (10 and 100 μmol/L) and verapamil (1 μmol/L), respectively. A real-time confocal microscope (FV1000, Olympus, Tokyo, Japan) was employed to obtain the fluorescent images. The image frame was continuously acquired every 1.107 s, and the images were stored in a high-speed hard disk. The artery rings were observed immediately after dye loading to acquire the fluorescent images of the resting state. Following the acquisition of resting response, the specimens were exposed to 50 mmol/L KCl and the images were acquired continuously[29]. The fluorescence intensity was calculated from individual image utilizing FV10-ASW (version 1.7, Olympus, Tokyo, Japan), and the changes of fluorescence intensity versus time were plotted. The change rate (%) of fluorescence intensity induced by KCl was calculated based on the formula below:

[(fluorescence intensity after exposure to KCl – fluorescence intensity before exposure to KCl)/fluorescence intensity before exposure to KCl]×100.

The change rate of fluorescence intensity reflected the change of $[Ca^{2+}]_i$.

Measurement of arterial pressure

SHRs weighing 250–300 g were divided into 5 groups (n=6–8 per group). Arterial pressure measurement was carried out via a non-invasive tail-cuff plethysmography method (CODA 6, Kent Scientific, Torrington, CT, USA)[30] once every day for a succession of five days to get the rats adapted to the operation. Right before the drug administration, the arterial pressure was measured. Then formononetin (5, 10, and 20 mg/kg), lacidipine (0.06 mg/kg) and saline containing 10% Tween 80 were injected via tail vein, respectively. The arterial pressure was assessed 0.5, 1, 2, 3, and 4 h after the administration.

Statistical analysis

Data are expressed as mean±SEM, and the differences between means were evaluated using SPSS for Windows 13.0 (SPSS, Chicago, IL, USA)[31]. Relaxant responses in each segment are expressed as a percentage of relaxation from the precontraction. E_{max} and R_{max} represent the maximal contraction and the maximal relaxation induced by vasoconstrictors and formononetin, respectively. Comparisons were made using 2-way ANOVA followed by Fischer's least significant difference *post-hoc* tests. Statistical significance was set at P<0.05. The EC_{50} was calculated by nonlinear regression analysis using the computer program GraphPad Prism 5 (San Diego, CA, USA).

Results

Relaxing responses of arteries to formononetin

KCl (60 mmol/L) or U46619 (1 μmol/L) was added to the baths to induce precontraction of the segments of rat mesenteric arteries, renal arteries, cerebral arteries, coronary arteries and abdominal aortas, respectively. The segments of rat mesenteric arteries, renal arteries and abdominal aortas were precontracted by phenylephrine (10 μmol/L). After sustained contraction was obtained, formononetin (1–300 μmol/L) was added cumulatively to the baths. Formononetin elicited a concentration-dependent relaxation in the rat artery segments pre-contracted by KCl (Figure 1A, Table 1), U46619 (Figure 1B, Table 2) or phenylephrine (Figure 1C, Table 3).

Table 1. Relaxation responses induced by formononetin in rat arterial segments pre-contracted by 60 mmol/L K^+; supplemental data for Figure 1A. Data are expressed as mean±SEM. n=6–8. ^bP<0.05, ^cP<0.01 *vs* cerebral artery. ^fP<0.01 *vs* coronary artery.

Artery	60 mmol/L K^+ (mN)	Formononetin		Control (DMSO)
		R_{max} (%)	EC_{50} (μmol/L)	R_{max} (%)
Mesenteric artery	7.19±0.42	96.2±1.0^b	14.9±1.8^{cf}	7.3±2.2
Abdominal aorta	9.01±0.71	96.7±1.7^b	19.1±4.1^{cf}	7.2±4.1
Renal artery	7.70±0.65	93.4±2.0	24.0±5.8	5.6±2.8
Coronary artery	5.31±0.56	91.8±5.8	36.3±3.5	4.8±2.1
Cerebral artery	5.30±0.52	86.1±3.9	37.9±4.9	7.2±6.1

R_{max}, maximum relaxation to formononetin or DMSO (control).

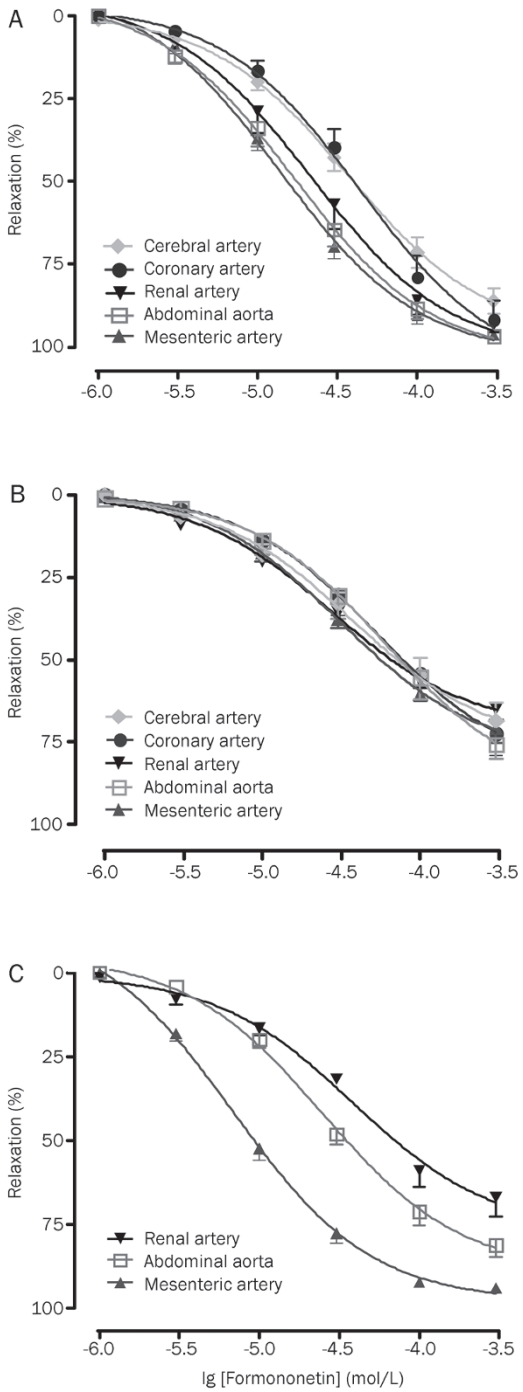

Figure 1. Cumulative concentration-response curves of formononetin. Rat isolated cerebral arteries, coronary arteries, renal arteries, abdominal aorta, and mesenteric arteries pre-contracted by 60 mmol/L K^+ (A) and 1 μmol/L U46619 (B), respectively; renal arteries, abdominal aorta, and mesenteric arteries pre-contracted by 10 μmol/L phenylephrine (C). Data are shown as mean±SEM. *n*=7–8 arteries.

Table 2. Relaxation responses induced by formononetin in rat arterial segments pre-contracted by 1 μmol/L U46619; supplemental data for Figure 1B. Data are expressed as mean±SEM. *n*=6–8.

Artery	1 μmol/L U46619 (mN)	Formononetin		Control (DMSO)
		R_{max} (%)	EC_{50} (μmol/L)	R_{max} (%)
Mesenteric artery	12.47±0.74	73.8±3.1	41.8±6.9	2.9±3.5
Abdominal aorta	13.88±0.70	75.8±4.3	59.8±17.0	6.0±2.2
Renal artery	10.65±0.63	65.0±3.3	33.8±6.5	2.9±2.0
Coronary artery	5.55±0.66	72.4±6.7	68.6±21.3	4.5±1.1
Cerebral artery	5.83±0.49	68.6±5.7	40.9±5.9	5.7±1.2

R_{max}, maximum relaxation to formononetin or DMSO (control).

Table 3. Relaxation responses induced by formononetin in rat arterial segments pre-contracted by 10 μmol/L phenylephrine (PE); supplemental data for Figure 1C. Data are expressed as mean±SEM. *n*=6–8. $^bP<0.05$, $^cP<0.01$ *vs* renal artery. $^eP<0.05$, $^fP<0.01$ *vs* abdominal aorta.

Artery	10 μmol/L PE (mN)	Formononetin		Control (DMSO)
		R_{max} (%)	EC_{50} (μmol/L)	R_{max} (%)
Mesenteric artery	7.96±0.70	94.0±1.5^{ce}	7.7±0.9^{cf}	8.1±2.1
Abdominal aorta	7.18±0.67	81.3±3.5^{b}	24.3±1.9^{c}	6.7±1.5
Renal artery	8.72±1.50	67.0±5.8^{e}	33.0±4.4^{e}	7.8±2.9

R_{max}, maximum relaxation to formononetin or DMSO (control).

Involvement of endothelium

Formononetin (1–300 μmol/L) concentration-dependently relaxed rat mesenteric artery segments pre-contracted by phenylephrine (10 μmol/L) with or without endothelium (Figure 2). Removal of endothelium suppressed the relaxing response to formononetin (R_{max}=96.25%±1.72% in endothelium-intact arteries, R_{max}=56.84%±1.89% in endothelium-denuded arteries, $P<0.05$), suggesting that the vasodilatation was partially endothelium-dependent.

Effects of NOS and cyclo-oxygenase (COX) inhibitors on the relaxing response to formononetin

Before treatment with formononetin in phenylephrine (10 μmol/L)-induced precontraction, rat mesenteric artery segments with endothelium were co-incubated with *L*-NAME (NOS inhibitor, 100 μmol/L), indomethacin (COX inhibitor, 10 μmol/L) or *L*-NAME (100 μmol/L)+indomethacin (10 μmol/L) for 20 min. Figure 2 showed that *L*-NAME and *L*-NAME+indomethacin attenuated the concentration-response curves of formononetin, while indomethacin lightly attenuated the concentration-relaxation curves of formononetin.

Effects of β-adrenoceptor and K^+ channels blockers on the relaxing response to formononetin

Before the addition of formononetin and 60 mmol/L KCl to the baths, the endothelium-denuded mesenteric artery segments were treated with propranolol (1 μmol/L), glibenclamide (10 μmol/L), tetraethylammonium (300 μmol/L) or bar-

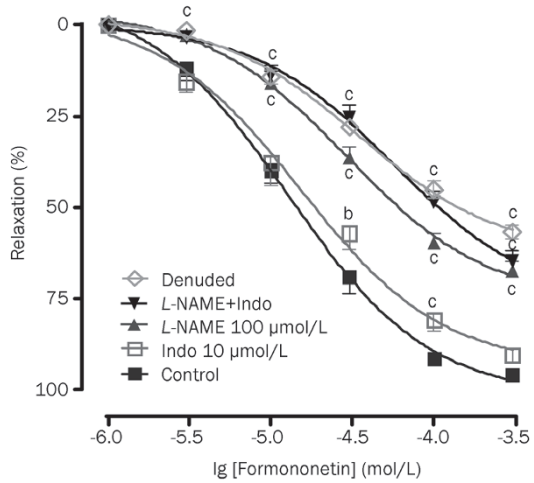

Figure 2. Effects of *L*-NAME (100 μmol/L), indomethacin (Indo, 10 μmol/L) and *L*-NAME+Indo (100 μmol/L+10 μmol/L) on the relaxing responses induced by formononetin in mesenteric arteries with intact endothelium, and effects of formononetin on mesenteric arteries with endothelium denuded and with endothelium intact (control). Rat isolated mesenteric arteries were pre-contracted by phenylephrine. Data are shown as mean±SEM. n=7–8 arteries. ^bP<0.05, ^cP<0.01 *vs* control.

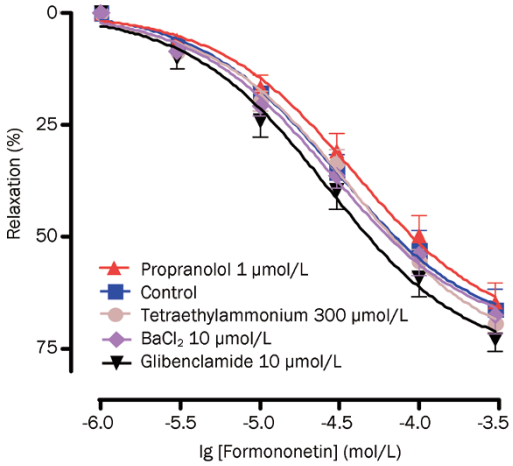

Figure 3. The vasorelaxant effect of formononetin in endothelium-free artery in the presence of propranolol (1 μmol/L), tetraethylammonium (300 μmol/L), $BaCl_2$ (10 μmol/L), or glibenclamide (10 μmol/L). Relaxation is expressed as a percentage of decrease in the maximal tension induced by 60 mmol/L K^+. Data are shown as mean±SEM. n=7–8 arteries. (control: in the absence of blockers)

ium chloride (10 μmol/L) for 20 min in order to test whether or not β-adrenoceptors, ATP sensitive K^+ channels, calcium-activated K^+ channels and inwardly rectifying K^+ channels were involved in the formononetin-induced relaxation. As shown in Figure 3, these blockers, compared with control (in the absence of blockers), did not shift the concentration-relaxation curves of formononetin significantly. No matter the blockers were present or not, the R_{max} and EC_{50} of formononetin in the endothelium-denuded artery segments showed no significant difference (P>0.05).

Effects of formononetin on K^+-induced contraction

Krebs solution was replaced with high KCl (10, 20, 40, and 80 mmol/L)-Krebs solutions 20 min after the treatment with DMSO (control) or formononetin (10, 30, and 100 μmol/L). The concentration-contraction curves of the endothelium-denuded rat mesenteric artery segments for KCl were constructed. Compared with control, formononetin shifted the concentration-contraction curves of KCl towards the right in a non-parallel manner (P<0.05, Figure 4A). The E_{max} of vasoconstrictive response to KCl was decreased and the EC_{50} value of KCl was increased by formononetin (P<0.05, Table 4).

Effects of formononetin on Ca^{2+}-induced contractions in Ca^{2+}-free depolarized Krebs solution

The endothelium-denuded rat mesenteric artery segments were exposed to Ca^{2+}-free and K^+-rich solution containing EDTA (100 μmol/L) and KCl (60 mmol/L) for 20 min. Then, $CaCl_2$ (0.01–10 mmol/L) was added cumulatively to the baths after treatment with DMSO (control) or formononetin (10, 30, and 100 μmol/L) for 15 min, and the concentration-response curves of $CaCl_2$ were constructed. Formononetin, compared with control, shifted the concentration-response curves of $CaCl_2$ towards the right in a non-parallel manner (P<0.05, Figure 4B). The E_{max} of vasoconstrictive response to $CaCl_2$ was decreased and the EC_{50} value of $CaCl_2$ was increased by formononetin (P<0.05, Table 4).

Effects of formononetin on phenylephrine- and 5-HT-induced contraction

The endothelium-denuded rat mesenteric artery segments

Table 4. EC_{50} values of different agonists contracting rat mesenteric arteries without endothelium after incubation with formononetin (For). Data are expressed as mean±SEM. n=6–8. ^bP<0.05, ^cP<0.01 *vs* control (DMSO).

Agonist		EC_{50} value			
		Control	For 10 μmol/L	For 30 μmol/L	For 100 μmol/L
KCl	mmol/L	30.1±0.95	31.7±0.6	32.7±0.8^b	38.0±0.8^c
$CaCl_2$	mmol/L	0.44±0.04	0.52±0.07	0.59±0.05^b	0.80±0.04^c
Phenylephrine	μmol/L	0.77±0.21	0.95±0.25	1.30±0.25	1.42±0.19^b
5-HT	μmol/L	0.77±0.14	1.14±0.23	1.23±0.18	2.13±0.09^c

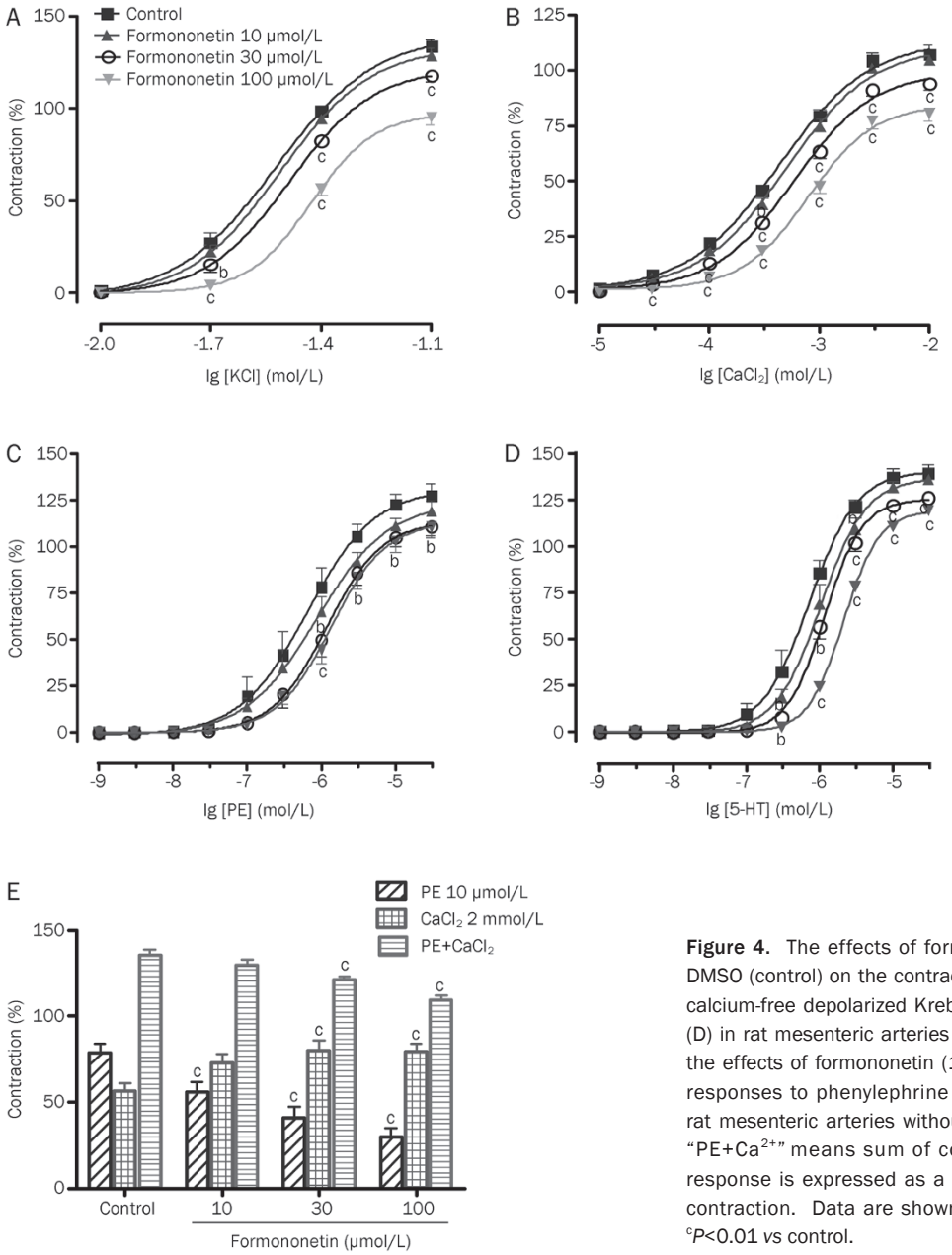

Figure 4. The effects of formononetin (10, 30, and 100 μmol/L) and DMSO (control) on the contractile responses to potassium (A), calcium in calcium-free depolarized Krebs solution (B), phenylephrine (C), and 5-HT (D) in rat mesenteric arteries without endothelium. Bar graph (E) shows the effects of formononetin (10, 30, and 100 μmol/L) on the contractile responses to phenylephrine (PE, 10 μmol/L) and $CaCl_2$ (2 mmol/L) in rat mesenteric arteries without endothelium in Ca^{2+}-free Krebs solution. "PE+Ca^{2+}" means sum of contractions to PE and Ca^{2+}. Contractile response is expressed as a percentage of the 60 mmol/L K^+ induced contraction. Data are shown as mean±SEM. n=8 arteries. $^bP<0.05$, $^cP<0.01$ *vs* control.

were pre-treated with DMSO (control) or formononetin (10, 30, and 100 μmol/L) for 20 min. Phenylephrine or 5-HT (0.001–30 μmol/L) was cumulatively added to the baths. Formononetin inhibited the phenylephrine- or 5-HT-induced vasoconstriction and concentration-dependently shifted the concentration-contraction curves towards the right in a non-parallel manner with a decreased E_{max} (Figure 4C, 4D). The EC_{50} of phenylephrine and 5-HT were increased by formononetin ($P<0.05$, Table 4).

Effects of formononetin on the contraction induced by phenylephrine dependent upon intracellular and extracellular calcium

The endothelium-denuded rat mesenteric artery segments were exposed to Ca^{2+}-free Krebs solution containing DMSO (control) or formononetin (10, 30, and 100 μmol/L) for 10 min in order to remove the extracellular Ca^{2+}, followed by addition of 10 μmol/L phenylephrine inducing phasic contractions caused by the release of intracellular Ca^{2+}. When the maximal contraction was obtained, $CaCl_2$ of 2 mmol/L was added to induce a tonic contraction evoked by the extracellular Ca^{2+} influx[32]. The results showed formononetin concentration-dependently inhibited the contraction induced by phenylephrine, but increased the $CaCl_2$-induced contraction (Figure 4E).

Effects of formononetin on the contraction induced by caffeine in Ca^{2+}-free solution

After the endothelium-denuded rat mesenteric artery segments were incubated with DMSO or formononetin (100

μmol/L) for 20 min in Ca^{2+}-free medium, artery contractions to caffeine (30 mmol/L) were obtained. The contractions induced by caffeine in the presence or absence of formononetin were 18.3%±1.8% and 18.0%±1.7% (contractile response is expressed as a percentage of the contraction induced by 60 mmol/L K^+), respectively. These data showed that formononetin did not affect the vasoconstriction induced by caffeine in Ca^{2+}-free solution (n=8 arteries, P>0.05).

Effects of formononetin on Ca^{2+} fluorescence intensity in rat mesenteric artery

Figure 5 presented the time course of the change rate of fluorescence intensity induced by 50 mmol/L KCl in rat mesenteric arteries. It could be seen that formononetin (10 and 100 μmol/L) concentration-dependently inhibited the change rate of fluorescence intensity.

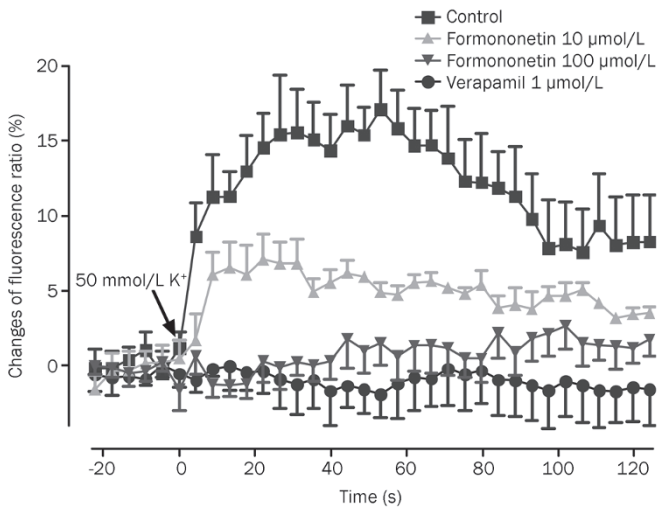

Figure 5. Effects of formononetin (10 and 100 μmol/L), verapamil (1 μmol/L), and DMSO (control) on Ca^{2+} fluorescence intensity in rat mesenteric arteries. The change rate of fluorescence intensity reflexes the change of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Each point represents the mean±SEM of five experiments.

Effects of formononetin on the SHR arterial pressure

The SHR arterial pressure was determined before drug administration. Then formononetin (5, 10, and 20 mg/kg), lacidipine (0.06 mg/kg) and saline containing 10% Tween 80 (control) were injected via tail vein. The arterial pressure was measured 0.5, 1, 2, 3, and 4 h after injection. It was found that formononetin lowered the systolic, diastolic and mean arterial pressures of the SHRs (Figure 6). The decline of arterial pressure reached the peak at 2 to 3 h after the treatment with formononetin, while the peak of the pressure decline induced by lacidipine was at 30 min after treatment.

Discussion

There have already been many reports about the antihyper-

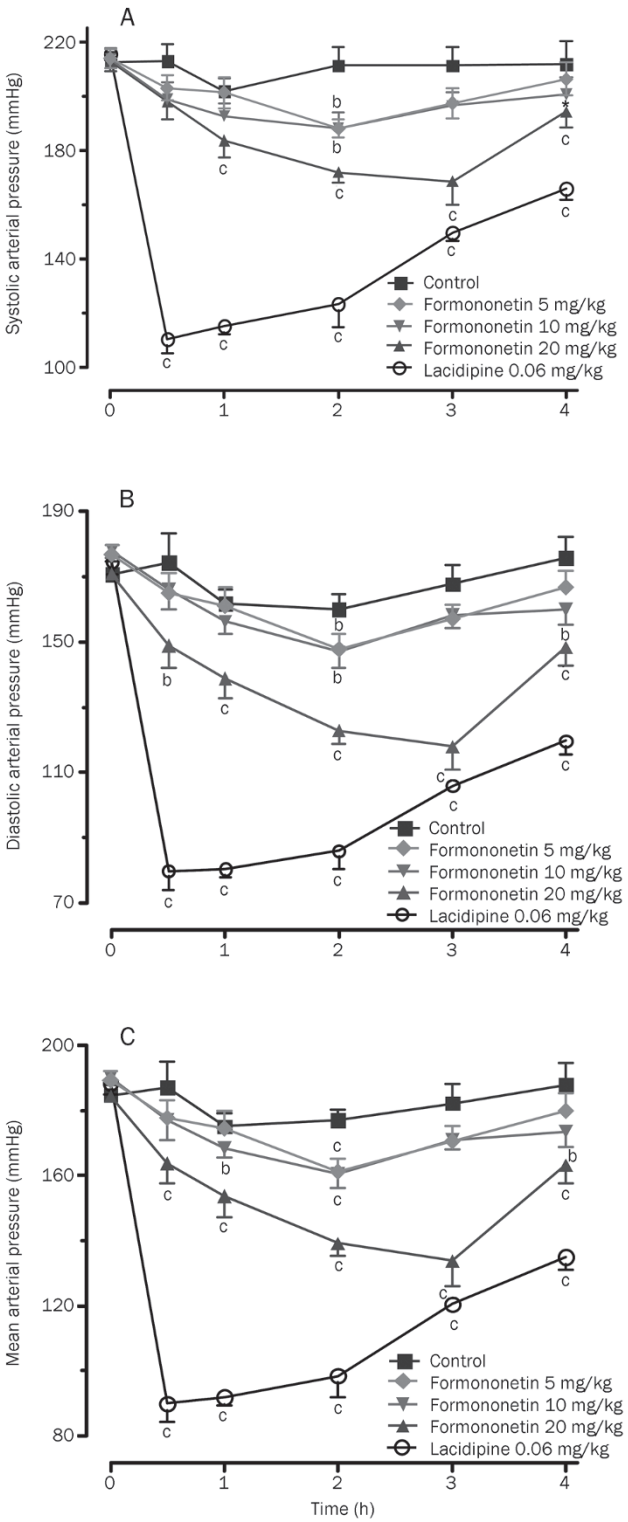

Figure 6. The effects of formononetin (5, 10, and 20 mg/kg), lacidipine (0.06 mg/kg), and saline containing 10% Tween 80 (control) on systolic arterial pressure (A), diastolic arterial pressure (B) and mean arterial pressure (C) of the SHRs. Formononetin and lacidipine were intravenously administered. Data are shown as mean±SEM. n=8. ^bP<0.05, ^cP<0.01 *vs* control.

tensive effects of some isoflavones[11]. However, no pharmacological or clinical study has been carried out to test the antihypertensive properties of formononetin. Our study first investigated the antihypertensive potential of formononetin, and the results show that formononetin can lower the arterial pressure in SHRs.

One of the key mechanisms of antihypertensive drugs is to lower the vascular resistance by directly dilating the blood vessels. Our experimental results show that formononetin can relax the arteries pre-contracted by vasoconstrictors, indicating that the antihypertensive activities of formononetin maybe stem from the vasorelaxant activity. This is in concert with the previous findings that formononetin relaxes rat isolated aorta[9] and some isoflavones have vasorelaxant activities[15]. We have found that mesenteric artery is more sensitive to formononetin than abdominal aorta and renal artery in precontraction by phenylephrine, and meanwhile mesenteric artery is one of peripheral arteries which contribute more resistance than aorta in hypertension, and therefore we chose to study the vasorelaxant mechanism of formononetin on mesenteric artery.

The vasorelaxant activity of antihypertensive drugs is achieved by influencing both vascular endothelial and vascular smooth muscle cells. Endothelium plays an important role in regulating the function of cardiovascular system. It synthesizes and releases several vasodilator substances, mainly including vasodilator PGs, NO, and EDHF[33]. The removal of endothelium markedly attenuates the relaxant response of arteries to formononetin, suggesting that the relaxation is endothelium-dependent. NO causes vascular relaxation by stimulating the production of cGMP and/or causing membrane hyperpolarization[34]. *L*-NAME, a NOS inhibitor, inhibits the relaxation of formononetin, indicating that the vasorelaxant activity of formononetin is related to NO release. Similar findings are reported elsewhere[9]. The vasorelaxant effect of PGs is realized through increasing intracellular cAMP levels[35]. We have found that indomethacin has little effect on the concentration-relaxation curves of formononetin, and therefore PG biosynthesis is little involved in the formononetin-induced relaxation. EDHF dilates arteries by increasing the membrane permeability of vascular smooth muscle cells to K^+[35]. The formononetin-induced relaxation in the mesenteric arteries without endothelium is not significantly different from that in the mesenteric arteries incubated with indomethacin+*L*-NAME, suggesting that other factors like EDHF are not involved in the endothelium-dependent relaxation. The relaxing response to formononetin has remained after the denudation of endothelial cells, indicating the involvement of endothelium-independent mechanism. Thus, it is conceivable that formononetin directly act on vascular smooth muscles as well as endothelial cells.

Vascular smooth muscle cells are also contributory to the vasorelaxant activity of antihypertensive drugs. β-adrenoceptors and K^+ channels are important regulators of arterial tone. β-adrenoceptor-induced relaxation is mediated by the increase of the intracellular cAMP concentration and/or activation of K^+ channels[36]. Opening of K^+ channels leads to membrane potential hyperpolarization and closure of voltage-dependent channels, which decreases Ca^{2+} entry and causes vasodilation[37]. In our experiments, propranolol, glibenclamide, tetraethylammonium and $BaCl_2$ did not affect the endothelium-independent relaxant response to formononetin in rat mesenteric arteries, suggesting that the K^+ channels and β-adrenoceptors are not involved in the vascular relaxation processes, which is in accordance with the previous findings that K^+ channel inhibitors did not affect the relaxation of genistein and zearalanone, two phytoestrogens, in rabbit coronary arteries[38]. By contrast, Wu *et al* proposed that formononetin caused opening of iberiotoxin-sensitive Ca^{2+}-activated K^+ channels and glibenclamide-sensitive adenosine triphosphate (ATP)-dependent K^+ channels in rat aorta[9]. This discordance may lie in the different responses of mesenteric artery and aorta to drugs[39].

There are two kinds of Ca^{2+} channels in smooth muscle cells: VDCC and receptor-operated Ca^{2+} channels (ROCC). KCl-induced contraction is due to membrane depolarization, leading to increased Ca^{2+} influx through VDCC. Formononetin inhibits the contraction induced by KCl in Krebs solution and $CaCl_2$ in high-K^+ depolarization medium, suggesting that formononetin may inhibit VDCC. This is in accordance with some previous findings[38].

Phenylephrine- or 5-HT-induced contraction in solution containing Ca^{2+} is due to activation of α_1-adrenoceptors or 5-HT receptors, which leads to an increase in intracellular calcium concentration via activation of the inositol phosphate cascade, releasing intracellular calcium and promoting the entry of extracellular calcium through ROCC[40, 41]. In the present study, formononetin treatment results in a rightward shift of concentration-contraction curves of phenylephrine or 5-HT in a non-parallel manner in medium with Ca^{2+}, suggesting that formononetin can inhibit ROCC or Ca^{2+} release from intracellular stores. Our further experiments show that formononetin inhibits the contraction induced by phenylephrine in Ca^{2+}-free solution while the tonic contraction evoked by the extracellular Ca^{2+} influx is increased, but the total contraction induced by $CaCl_2$ and phenylephrine is decreased by formononetin. These results suggest that formononetin inhibits intracellular Ca^{2+} release from Ca^{2+} stores in vascular smooth muscle cells, but does not inhibit the extracellular Ca^{2+} influxing through ROCC. This is partly consistent with some previous findings[42]. The increased contraction induced by $CaCl_2$ may be a compensatory mechanism of suppressed Ca^{2+} release. Thus, it is likely that Ca^{2+} mobilization in smooth muscle cells is inhibited by formononetin.

The release of intracellular Ca^{2+} is mainly regulated by ryanodine receptor system and inositol 1,4,5-trisphosphate (IP3) receptor system. The former may function through a Ca^{2+}-induced Ca^{2+} release mechanism when the receptors are activated by caffeine[43]. The latter induces Ca^{2+} release directly when the receptors are bound to IP3. Furthermore, formononetin does not affect the caffeine-induced contraction in endothelium-denuded arteries, which rules out the possible involvement of ryanodine receptors. Therefore, it is likely that

the IP3 receptor contributes to Ca^{2+} release.

Laser scanning confocal microscope and fluorescent probe are widely employed to monitor dynamic changes of $[Ca^{2+}]_i$ in various cells and tissues[29]. Our finding that formononetin inhibits the KCl-induced increase of $[Ca^{2+}]_i$ in rat mesenteric artery further proves that formononetin decreases intracellular calcium.

In summary, formononetin, with antihypertensive potential, has multiple targets and a moderate effect in lowering arterial pressure, which is different from typical antihypertensive drugs. Besides, being a natural compound, formononetin may have little toxic effect. What's more, formononetin possesses antioxidant property[44], and it increases the expression of eNOS[9] and lowers blood lipid[45], which will be beneficial in preventing and treating the complications of hypertension, such as cardiac and vascular remodeling, lipid abnormalities and atherosclerosis. Therefore, formononetin may be of value in treating and preventing hypertension.

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Author contribution

Tao SUN and Yong-xiao CAO designed the research; Tao SUN and Rui LIU performed the experiments; and Tao SUN and Yong-xiao CAO analyzed the data and wrote the paper.

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