Phytoestrogen genistein decreases contractile response of aortic artery *in vitro* and arterial blood pressure *in vivo*

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KEY WORDS genistein; aorta; smooth muscle; muscle relaxation; calcium channel; vascular endothelium; blood pressure

ABSTRACT

AIM: To determine the mechanisms of effects of phytoestrogen genistein on the contracted rabbit aortic arteries *in vitro*, and observe the effect of genistein and 17-β estradiol on mean arterial pressure (MAP) in ovariectomized (OVX) rats. **METHODS:** (1) Strips of rabbit aortic smooth muscle were suspended in organ baths containing Kreb's solution, and then isometric tension was measured. (2) Female mature Wistar rats underwent a bilateral ovariectomy (OVX). Sham-operated rats (SHAM) were used as controls. After administration of genistein (0.4 $mg \cdot kg^{-1} \cdot d^{-1}$, sc), 17-β estradiol (1 $mg \cdot kg^{-1} \cdot d^{-1}$, sc) or their vehicle sesame oil for 21 d, MAP was measured. **RESULTS:** (1) Similar to 17-β estradiol, genistein could dose-dependently relax 40 mmol/L KCl-precontracted arterial strips. Incubation with N^{ω}-*L*-nitro-arginine (*L*-NNA), methylene blue (MB), indomethacin, propranolol or endothelium removal did not affect relaxation induced by genistein. In calcium-free solution containing 0.01mmol/L egtazic acid (EGTA), genistein inhibited not only the first phase contraction induced by noradrenaline (NA), but also the second contraction induced by $CaCl_2$. In addition, genistein could reduce the contractile responses of NA, KCl and $CaCl_2$, and shift their cumulative concentration-response curves rightward. (2) MAP in OVX rats was significantly higher compared with that of SHAM rats. However, after chronically treatment with genistein or 17-β estradiol for 21 d the baseline MAP in OVX rats was reduced significantly. **CONCLUSIONS:** (1) The vasodilator effect of genistein *in vitro* is endothelium independent and not related to the nitric oxide, its mechanisms being probably due to inhibition of Ca^{2+} influx through calcium channels in a noncompetitive manner and Ca^{2+} release from intracellular store induced by NA. (2) Administration of genistein or 17-β estradiol can chronically decrease MAP in OVX rats.

INTRODUCTION

Phytoestrogens are naturally found in many plants, particularly soy beans, and they are defined as plant substances that are structurally or functionally similar to estradiol[1,2]. Genistein, a phytoestrogen, may have estrogenic cardioprotective actions[3]. Epidemiological data suggest a reduction in the incidence of coronary heart disease in humans who have a high intake of phytoestrogens[4]. Increased plasma levels of the phytoestrogen genistein are suggested as an explanation for the infrequency of hot flashes and menopausal symptoms in women[5,6]. The affinity of genistein for the classic estrogen α receptor presented on reproduc-

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tive organ is less than that of estrogen[7]. However, genistein has a similar affinity as estrogen for the novel estrogen β receptor in the vasculture. Many studies demonstrate that *in vitro* 17-β estradiol has a direct vasodilatory action which may partly contribute to its cardiovascular protection[8,9]. Recently there are evidences that phytoestrogen genistein also can be vasoactive, it enhances coronary vasoreactivity in macaque monkeys[10], relax mesenteric and coronary arteries *in vitro*[11,12], and also relax human forearm vasculature *in vivo*[13]. However the mechanism involved its vasodilator effect is not completely understood, and it is uncertain whether genistein can decrease blood pressure. Therefore, we assessed the relationship between vasorelaxation induced by genistein with Ca^{2+} influx, intracellular Ca^{2+} release, nitric oxide (NO), cyclic guanine monophosphate (cGMP), prostaglandins and adrenergic β receptors. We also investigated the effect of genistein and 17-β estradiol on mean arterial pressure (MAP) in ovariectomized (OVX) rats.

MATERIALS AND METHODS

Drugs Genistein, 17-β estradiol, N^{ω}-*L*-nitro-arginine (*L*-NNA) (Sigma, Chemical Co, USA); acetylcholine (ACh) and propranolol (The Second Pharmaceutical Factory of Beijing, China); indomethacin (Jiangsu Taicang Pharmaceutical Co, China); noradrenaline (NA, Datong Huida Pharmaceutical Factory, China); methylene blue (MB, Chemical Industry of Antichemical College, China). Genistein and 17-β estradiol were dissolved in 1 % dimethylsulfoxide (Me_2SO) *in vitro* experiment or in sesame oil *in vivo* experiment. Indomethacin was dissolved in a Na_2CO_3 solution at pH 7.4.

Arterial tension studies Adult male or nonpregnant female rabbits (general grade, certificate No 14-004) weighing 2.50±0.35 kg were purchased from Experimental Animal Center of Lanzhou Biological Productive Institute and all animal experiments were approved by the College Committee on the Use and Care of Animals. The rabbits were killed by stunning and exsanguinations. The thoracic aorta was rapidly removed and carefully cleaned of connective tissue and blood, then cut into spiral strip with 10 mm long and 3 mm wide. Each strip was suspended in a tissue chamber containing 37 ºC Krebs solution composed of (mmol/L) NaCl 120, KCl 5.9, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $NaHCO_3$ 15.4, $CaCl_2$ 2.5, and glucose 11.5, bubbled with 95 % O_2 and 5 % CO_2. Isometric tension generated by vascular smooth muscle was measured using a force transducer (JH-2) and recorded with BL-410 Experimental System of Biological Function (TME, China) through IBM computer and 2D ink-writing recorders (Chengdu Instrument Plant). Resting tension was set to 2 g. After 90 min of equilibration, the strips were activated with 1 μmol/L noradrenaline (NA) to check their contractility.

In some strips, the endothelium was removed by gentle rubbing with a cotton lot. In all tissues the presence or absence of a functionally intact endothelium was tested by measuring the ability of acetylcholine (1 μmol/L) to produce relaxation of tissues precontracted with NA (1 μmol/L).

After equilibration, various experiments were done.

(1) The strips were precontracted with 40 mmol/L KCl, when the contractile response had reached a stable plateau, genistein (1-500 μmol/L), 17-β estradiol (1-500 μmol/L) or the same volume of solvent were added in progressively increasing cumulative concentrations every ten minutes.

(2) In some experiments, aortic strips with or without endothelium were treated with 40 mmol/L KCl. When the contractile response reached a plateau (approximately 15-20 min), genistein (250 μmol/L) or equivalent solvent was added. After 20 min, strips were washed with normal Krebs' solution, and then strips with endothelium were once again treated with KCl and the response to genistein was measured after preincubation with one of the following substances: 100 μmol/L N^{ω}-*L*-nitro-arginine (*L*-NNA), 10 μmol/L methylene blue (MB), 10 μmol/L indomethacin, and 10 μmol/L propranolol.

(3) To evaluate the possible effect of genistein on NA-induced calcium release and calcium influx through receptor-operated calcium channels (ROCs), aortic strips denuded of endothelium were incubated in calcium-free solution containing 0.01 mmol/L egtazic acid (EGTA) for 30 min, and then treated with NA (1 μmol/L). When the contractile response had reached a plateau, $CaCl_2$ (10 mmol/L) was added into the organ chamber and a further contraction was obtained. Tissues were washed with Ca^{2+}-free solution and left to return to baseline tone. The strips were then treated with NA and $CaCl_2$ again after being incubated with genistein (500 μmol/L) or the solvent.

(4) Strips were stabilized at 2 g resting tension for 90 min in Krebs' solution and the concentration-response curve to NA (0.03-10 μmol/L) was then obtained. After washout of NA, the experiment was repeated in the presence of genistein (100 or 500 μmol/L).

(5) The concentration-response curve to KCl (10-100 mmol/L) was observed in the absence or presence of genistein (50, 100 or 500 μmol/L).

(6) Aortic strips were incubated in calcium-free solution containing 0.01 mmol/L EGTA for 60 min. The calcium concentration-dependent contraction curve was then measured in K^+ depolarization medium (40 mmol/L KCl). After washed with calcium-free solution, the strips were incubated with genistein (10 μmol/L) or equivalent solvent for 20 min and the calcium concentration-dependent contraction curve was then obtained again.

Arterial blood pressure measurement Female mature Wistar rats (grade II, certificate No 14-006) weighing 214.07±4.85 g underwent a bilateral ovariectomy (OVX). Sham-operated rats (SHAM) were used as control. One week after surgery, the OVX animals received daily subcutaneous injections of genistein (0.4 mg/kg), 17-β estradiol (1 mg/kg) or injections of equivalent volume sesame oil at the same time. After 21 d, the rats were anesthetized with urethan (1 g/kg, ip). Polyethylene catheters were inserted into the external jugular vein for drug administration and into the common carotid artery for mean arterial pressure (MAP) measurement respectively. The arterial catheter was connected to a pressure transducer, and the blood pressure was displayed on the channel of BL-410 Experimental System of Biological Function (TME, China) through IBM computer. The changes of resting arterial blood pressure in different group rats was assessed.

Data analysis All results are expressed as mean±SD. Relaxation was expressed as percentage relaxation of contraction induced by KCl (40 mmol/L). In experiments involving a concentration-response curves, the results were expressed as percentage of control contractile response induced by 10 μmol/L NA, 100 mmol/L KCl and 10 mmol/L $CaCl_2$ respectively. The EC_{50} values were calculated using a computer statistical package (SPSS 10.0 for Windows). Statistical analysis was performed using *t*-test and analysis of variance (ANOVA). Each group was compared with the solvent control. A probability level (*P* value) of less than 0.05 was considered significant.

RESULTS

Relaxing effects of genistein and 17-β estradiol on KCl-precontracted aortic arteries In KCl precontracted endothelium-intact arterial strips, phytoestrogen genistein and 17-β estradiol could dose-dependently induce a relaxation response (r=0.99, P<0.001)

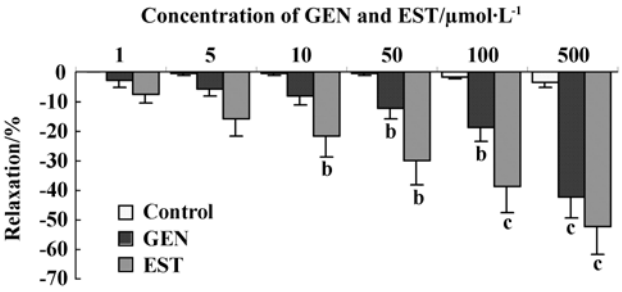

Fig 1. Relaxant effects of genistein (GEN) and 17-β estradiol (EST) on endothelium-intact rabbit aortic strips precontracted with 40 mmol/L KCl. n=8. Mean±SD. ^bP<0.05, ^cP<0.01 *vs* control.

when compared to the solvent control (Fig 1).

Effects of *L*-NNA, MB, endothelium removal, indomethacin and propranolol on responses to genistein in KCl-treated strips Genistein (250 μmol/L) caused 27.76 %±2.66 % relaxation of KCl-treated strips (P<0.01, n=17). After incubation with *L*-NNA (100 μmol/L), MB (10 μmol/L), indomethacin (10 μmol/L), propranolol (10 μmol/L) or endothelium removal, percentages of relaxation induced by genistein (250 μmol/L) were 26.50 %±2.46 %, 28.77 %±4.68 %, 26.84 %±3.28 %, 33.71 %±5.41 %, and 30.22 %±4.34 %, respectively, and no obvious differences were found between them (all P>0.05, n=8).

Effects of genistein on biphasic contraction induced by NA and $CaCl_2$ In calcium-free (0.01 mmol/L EGTA) Krebs' solution, NA (1 μmol/L) caused a transient contraction (1.02±0.32) g. As soon as such contraction reached a plateau, 10 mmol/L $CaCl_2$ was rapidly added into the bath and another higher contractile response occurred (1.95±0.74 g, n=11). Genistein (250 μmol/L) reduced the first contraction induced by NA from 1.02±0.32 g to 0.38±0.18 g (P<0.01, n=11), and also reduced the second contraction caused by $CaCl_2$ from 1.95±0.74 g to 1.60±0.84 g (P<0.05, n=11, Fig 2) in Ca^{2+}-free solution.

Inhibitions of genistein on NA and KCl concentration-dependent contractile responses NA (0.03-10 μmol/L) and KCl (10-100 mmol/L) elicited concentration-dependent contractions in isolated aortic strips. However, genistein (50, 100, or 500 μmol/L) significantly reduced the responses to NA and KCl, and made their concentration-dependent contraction curves shifted to the right in a dose-dependent manner (Fig 3 and 4). The EC_{50} values in control and after treatment with genistein (100 or 500 μmol/L) were 1.51±0.30 μmol/L,

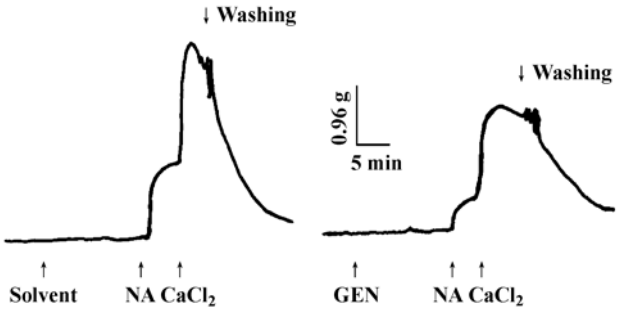

Fig 2. Effects of genistein (GEN, 250 μmol/L) on contractile responses induced by NA and $CaCl_2$.

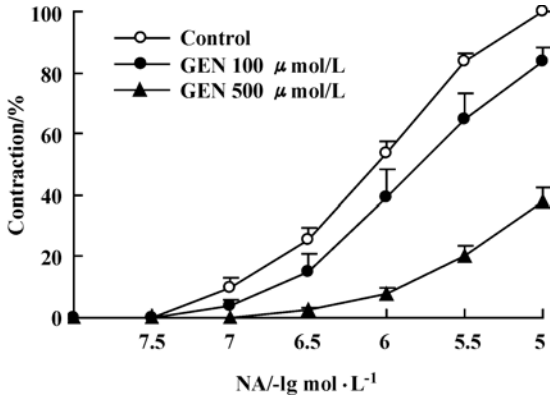

Fig 3. Effects of genistein (GEN) (100 or 500 μmol/L) on NA-induced contraction of rabbit aorta. Data are expressed as percentage of maximal contraction induced by NA in controls. *n*=6. Mean±SD.

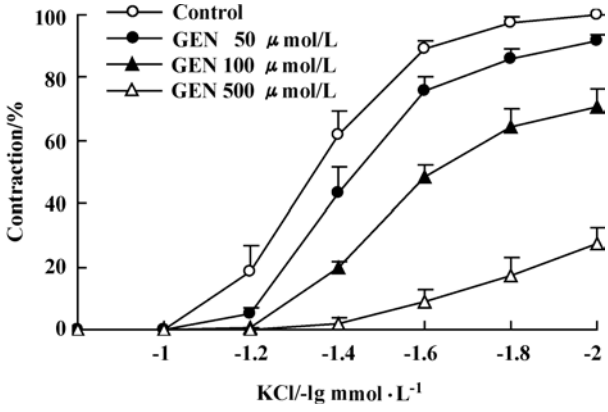

Fig 4. Effects of genistein (GEN) (50, 100, or 500 μmol/L) on KCl-induced contraction of rabbit aorta. Data are expressed as percentage of maximal contraction induced by KCl in controls. *n*=8. Mean±SD.

4.19±2.16 μmol/L (*n*=6, *P*<0.01 *vs* control) and 11.55±2.11 μmol/L (*n*=6, *P*<0.01 *vs* control) for NA respectively. The EC_{50} values in control and after treatment with genistein (50, 100, or 500 μmol/L) were (26.03±5.90) mmol/L, (38.80±8.70) mmol/L, (62.92±15.47) mmol/L (*n*=8, *P*<0.05 *vs* control) and (124.11±23.68) mmol/L (*n*=8, *P*<0.05 *vs* control) for KCl respectively.

Inhibition of genistein on calcium concentration-dependent curve In K^+(40 mmol/L) depolarized tissues in Ca^{2+}-free medium, pretreatment with genistein (10 μmol/L) led to a reduction in the contractile response to $CaCl_2$, shifting the concentration-response curve to the right (Fig 5). The EC_{50} values of calcium for control and 10 μmol/L genistein were (1.06±0.51) mmol/L and (6.09±1.93) mmol/L respectively (*n*=6, *P*<0.05 *vs* control).

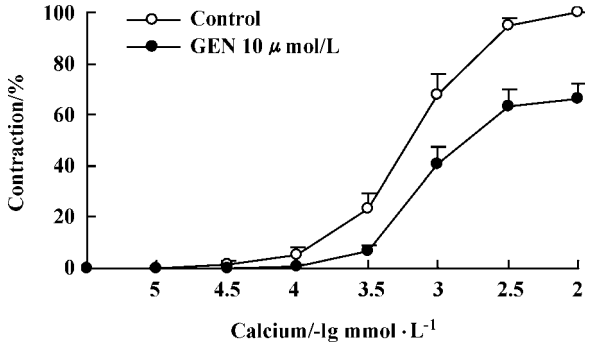

Fig 5. Effect of genistein (GEN, 10 μmol/L) on calcium concentration-dependent contraction curve in rabbit aorta. Data are expressed as percentage of maximal contraction induced by calcium in controls. *n*=6. Mean±SD.

Effects of genistein and 17-β estradiol on mean arterial pressure in OVX rats MAP in OVX rats was significantly higher compared with that of ovary-intact rats. However , after chronically treatment with genistein and 17-β estradiol for 21 d the baseline MAP in OVX rats (*P*<0.05, *n*=12) reduced significantly (Fig 6).

DISCUSSION

We have shown that the phytoestrogen genistein induced significant relaxation of aortic artery strips in the similar way as 17-β estradiol did. The relaxation was dose-dependent. The reports in effects of NO and endothelial cell on vasorelaxation induced by genistein are not consistent. In present experiment, no difference was observed between strips with or without endothelium. Preincubation with *L*-NNA, an inhibitor of NO synthesis, did not affect genistein-induced aortic artery relaxation. Methylene blue, an inhibitor of cGMP synthesis[14] and an important second messenger mediating vascular relaxation, also did not affect

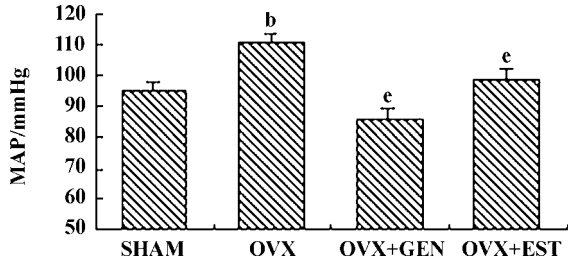

Fig 6. Effect of genistein (GEN, 0.4 mg·kg^{-1}·d^{-1}) and 17-β estradiol (EST, 1 mg·kg^{-1}·d^{-1}) on mean arterial pressure (MAP) in OVX rats. MAP is shown in the following groups: Sham-operated rats (SHAM), ovariectomized (OVX), and OVX rats treated chronically with genistein (OVX+GEN) and 17-β estradiol (OVX+EST) for 21 d. *n*=12. Mean±SD. $^{b}P<0.05$ *vs* corresponding SHAM; $^{e}P<0.05$ *vs* corresponding OVX.

the relaxation induced by the phytoestrogen. Our results suggest that the relaxation of the rabbit aorta caused by genistein is independent on NO and on cGMP. Just as Marsh[15] reported that this is of therapeutic interest, as conceivably genistein might be used in patients with diseased and dysfunctional endothelium.

The endothelium might release vasoconstrictor and vasodilator prostaglandins. In our experiments, indomethacin did not affect the relaxation induced by genistein in the intact aorta. Propranolol, an antagonist of adrenergic β receptors, also did not affect vasorelaxation. These results indicate that the release of vasodilator prostanoids and adrenergic β receptors are not involved in aortic relaxation induced by genistein.

The vasoconstrictive responses to KCl are usually used to assess the contractile ability of the vascular smooth muscle[16]. Potential dependent calcium channels (PDCs) are activated by depolarization of the plasma membrane when the extracellular K^+ concentration is increased. In our experiment, genistein relaxed KCl-precontracted aortic strips, and incubation with genistein not only shifted the KCl concentration-dependent contraction curves to the right in normal Krebs' solution but also moved the calcium concentration-dependent contraction curves to the right in high K^+ depolarization medium, and inhibited KCl concentration-dependent contractile responses in a noncompetitive manner. These results are consistent with the effect of 17-β estradiol on vessels as we reported previously[9] and supported by the other study[12]. In present study, our results suggest that genistein may have Ca^{2+} antagonistic properties and can inhibit extracellular Ca^{2+} influx through PDC.

Noradrenaline (NA) can activate receptor-operated calcium channels (ROCs) in the cellular membrane of vascular smooth muscle and increase calcium influx, while also activating G proteins and phospholipase C to produce inositol trisphosphate (IP_3) which causes calcium release from endoplasmic reticulum[17]. In present experiment, genistein could decrease the concentration-dependent responses induced by NA, suggesting that genistein probably can inhibit Ca^{2+} influx via ROCs or Ca^{2+} release induced by IP_3, but which one of them involved in the inhibition of contraction caused by NA is not completely understood. As we know, the contractile response to a number of agonists including NA comprises two distinct components in Ca^{2+}-free medium: an initial phasic component that results from IP_3-mediated release of Ca^{2+} from intracellular Ca^{2+} stores followed by a tonic component that requires addition of Ca^{2+} in the continuous presence of the agonist, due to Ca^{2+} influx[18]. This is so-called biphasic contraction induced by NA and Ca^{2+}. We found that genistein not only attenuated NA-induced contraction but also decreased latter $CaCl_2$-induced contraction significantly. These results indicate that the inhibitions of Ca^{2+} release and calcium influx induced by NA are all involved in the mechanisms of vascular smooth muscle relaxation caused by genistein.

The experiment *in vivo* showed that in OVX rats, compared with sham-operated animal, mean artery blood pressure (MAP) significantly increased, 17-β estradiol significantly reverted the baseline MAP. This result is the same as a previous study[19]. However, the major finding of the present study is that treatment with phytoestrogen genistein can also decrease MAP in OVX rats, which probably give a reasonable explanation for Washburn's study[20] that soy supplementation in the diet of nonhypercholesterolemic, nonhypertensive and perimenopausal women resulted in significant decline in diastolic blood pressure.

In conclusion, phytoestrogen genistein can decrease contractile response of rabbit aortic artery *in vitro* and arterial blood pressure of OVX rats *in vivo* in the same way as 17-β estradiol. The relaxation *in vitro* is endothelium-independent, and not related to NO, cGMP, the release of vasodilator prostanoids and adrenergic β receptor. The mechanism probably involves in the inhibitions of Ca^{2+} influx through calcium channels in a noncompetitive manner and Ca^{2+} release from intracellular stores induced by NA. Therefore, genistein has a similar vasoactivity as 17-β estradiol does, and

has a potential to replace estradiol in the prevention and treatment of vascular diseases.

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