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Effects of phytoestrogens resveratrol and phloretin on contractile response of aortic strips in rabbits

LI Hong-Fang[1*], WANG Long-De[2], TIAN Zhi-Feng[3], LI Wei[1], ZHENG Tian-Zhen[1]
(1. *Department of Physiology*, 3. *Lab of Functional Medicine*, *College of Basic Medicine*, *Lanzhou University*, *Lanzhou* 730000, *China*; 2. *Affiliated Hospital*, *Gansu Chinese Traditional Medical College*, *Lanzhou* 730020, *China*)

Abstract: **AIM** To investigate whether the relaxation characteristics of phytoestrogens resveratrol and phloretin on contractile response of aortic strips are similar to that of estrogen and the mechanisms underground. **METHODS** Aortic strips from rabbits were suspended in organ baths containing Krebs solution, and then isometric tension was measured. **RESULTS** Resveratrol and phloretin inhibited the contractile responses to norepinephrine (NE), KCl and $CaCl_2$, shifted their concentration-response curves rightward with pD_2' values of 2.89, 3.34, 3.37 for resveratrol and 3.23, 3.52, 3.77 for phloretin respectively. Also both of them concentration-dependently relaxed KCl-precontracted aortic strip. The relaxing response of resveratrol but not of phloretin in aortic strip was significantly reduced by removal of endothelium or incubation with N^{ω}-*L*-nitro-arginine and methylthioninium chloride, however both their relaxant effects were not affected by indometacin and propranolol. In Ca^{2+}-free Krebs solution containing 0.01 mmol·L^{-1} EGTA, resveratrol and phloretin inhibited NE-induced contraction which was caused by Ca^{2+} release from intracellular store, but did not affect the contraction which was induced by Ca^{2+} influx. **CONCLUSION** Resveratrol and phloretin can induce vasorelaxations which may relate to inhibition of Ca^{2+} influx through potential-dependent calcium channels and Ca^{2+} release from intracellular stores, and the relaxing response of resveratrol is endothelium-dependent in part, but of phloretin is not endothelium-dependent.

Key words: phytoestrogens; resveratrol; phloretin; muscle, smooth, vascular; aorta, thoracic; vasodilation; endothelium, vascular

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Many studies have demonstrated that estrogen can produce direct vasorelaxing actions[1,2], and this may partly contribute to the cardiovascular protective effects[3]. Resveratrol and phloretin are naturally found in plants and are defined as phytoestrogens because their structure and function are similar to estrogen[3]. Epidemiological data suggest a reduction in the incidence of coronary heart disease in humans who have a high intake of phytoestrogen[3]. Using competitive binding techniques with [^{3}H]17β-estradiol and estrogen receptor in cell-free extracts, it has showed that hydroxylated flavonoid phloretin can interact directly with the estrogen receptor[3]. There is evidence as well that resveratrol exhibits variable degrees of estrogen receptor agonism in different test system[4]. Despite the increasing interest in the effects of phytoestrogens on cardiovascular system, it is unknown whether they share the same vasodilator characteristics of estrogen. Therefore, it is necessary to discuss the effect of resveratrol and phloretin on vascular contractility, which could contribute to the cardiovascular protective effect of phytoestrogens. The possible role of the endothelium, cGMP, NO, prostaglandins, β-adrenergic receptor, calcium influx and calcium release from intracellular stores on

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Biography: LI Hong-Fang (1966 –), female, native of Lanzhou, Gansu Province, PhD, and main research field is physiology and pharmacology of smooth muscle.
* Corresponding author. E-mail: lihf@lzu.edu.cn Tel: 86-931-8289109

resveratrol- and phloretin-induced vasorelaxation were also examined in this study.

1 MATERIALS AND METHODS

1.1 Drugs

The following drugs were used: resveratrol, phloretin, N^{ω}-*L*-nitro-arginine (*L*-NNA) (Sigma, USA); acetylcholine (ACh) and propranolol (The Second Pharmaceutical Factory of Beijing, China); indometacin (Jiangsu Taicang Pharmaceutical Co, China); norepinephrine (NE, Datong Huida Pharmaceutical Factory, China); methylthioninium chloride (MC, Merck, Germany). Resveratrol and phloretin were prepared by dissolving in dimethylsulfoxide. Indometacin was dissolved in a Na_2CO_3 solution at pH 7.4.

1.2 Arterial tension studies

Adult male and nonpregnant female rabbits weighing 2.5 – 3.0 kg were purchased from Animal Center of Lanzhou Medical College and all animal experiments were approved by the College Committee on the Use and Care of Animals. Rabbits were sacrificed by stunning and exsanguinations. The thoracic aorta was rapidly removed and carefully cleaned of connective tissue and blood, then cut into spiral strips that were 10 mm long and 3mm wide. Each strip was suspended in a 5 mL tissue chamber containing 37℃ Krebs solution composed of (mmol·L^{-1}) 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 ink-writing recorder (LMS-2B, Chendu, China) and BL-420E$^+$ Experimental System of Biological Function (TME, China) through IBM computer. Resting tension was set to 2 g. After 90 min of equilibration, the strips were activated with 1 μmol·L^{-1} NE to check their integrity.

In some strips, the endothelium was removed by gentle rubbing with a cotton lot. The absence of a functionally intact endothelium of the strip was demonstrated by unresponsiveness to 1 μmol·L^{-1} ACh in the strip precontracted with NE (1 μmol·L^{-1}).

After equilibration, various experiments were done: ① The strips were precontracted with 40 mmol·L^{-1} KCl, when the contractile response had reached a stable plateau, resveratrol and phloretin were added into the chamber to progressively increase the cumulative concentration (1 – 500 μmol·L^{-1}) every 10 min. ② In some experiments, aortic strips with or without endothelium were treated with 40 mmol·L^{-1} KCl. When the contractile response reached a plateau (approximately 15 – 20 min), resveratrol, phloretin (250 μmol·L^{-1}) or equivalent solvent was added and relaxation response was recorded. After washout, strips with endothelium were once again treated with KCl and the response to resveratrol and phloretin was measured after preincubation with one of the following substances for 15 min: 100 μmol·L^{-1} *L*-NNA, 10 μmol·L^{-1} MC, 10 μmol·L^{-1} indometacin or 10 μmol·L^{-1} propranolol. ③ To evaluate the possible effect of resveratrol and phloretin on NE-induced calcium release and calcium influx through receptor-operated calcium channels (ROC), denuded aortic strips were incubated in calcium-free solution containing 0.01 mmol·L^{-1} EGTA for 30 min, and then treated with NE (1 μmol·L^{-1}). When the contractile response had reached a plateau, $CaCl_2$ (10 mmol·L^{-1}) 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 by NE and $CaCl_2$ again before being incubated with resveratrol and phloretin (250 μmol·L^{-1}) or solvent for 20 min. ④ Strips were stabilized at 2 g resting tension for 90 min in Krebs solution and the concentration-response curve to NE (0.03 – 10 μmol·L^{-1}) was then obtained. After washout, the experiment was repeated in the presence of resveratrol and phloretin (100 and 500 μmol·

L^{-1}). ⑤ The concentration-response curve to KCl(10 – 100 mmol·L^{-1}) was observed in the absence and presence of resveratrol and phloretin (50, 100 and 500 μmol·L^{-1}). ⑥ Aortic strips were incubated in calcium-free solution containing 0.01 mmol·L^{-1} EGTA for 60 min. The calcium concentration-dependent contraction curve was then measured in K^+ depolarization medium (40 mmol·L^{-1} KCl). After washing with calcium-free solution, the strips were incubated with resveratrol (250 μmol·L^{-1}) and phloretin (100 μmol·L^{-1}) for 20 min and the calcium concentration-dependent contraction curve was then obtained again.

1.3 Data analysis

All results were expressed as $\bar{x} \pm s$. Relaxation was expressed as percentage relaxation of contraction induced by KCl (40 mmol·L^{-1}). In experiments involving concentration-response curves, the results were expressed as percentage of control contractile response induced by 10 μmol·L^{-1} NE, 100 mmol·L^{-1} KCl and 10 mmol·L^{-1} $CaCl_2$ respectively. Statistical analysis was performed using analysis of variance (ANOVA) and *t*-test. The pD_2' values were calculated using the method provided by Xu, *et al*[5].

2 RESULTS

2.1 Relaxant effects of resveratrol and phloretin on KCl-precontracted aortic strips

Resveratrol and phloretin caused concentration-dependent relaxation of endothelium-intact aortic strips precontracted with 40 mmol·L^{-1} KCl (both $r = 0.98$, $P < 0.001$) as compared to the solvent control (Fig 1).

2.2 Effects of *L*-NNA, MC, endothelium removal, indometacin and propranolol on responses to resveratrol and phloretin in KCl-treated strips

As shown in Fig 2, incubation with *L*-NNA (100 μmol·L^{-1}), MC (10 μmol·L^{-1}) or endothelium removal partially reduced the relaxation induced by resveratrol (250 μmol·

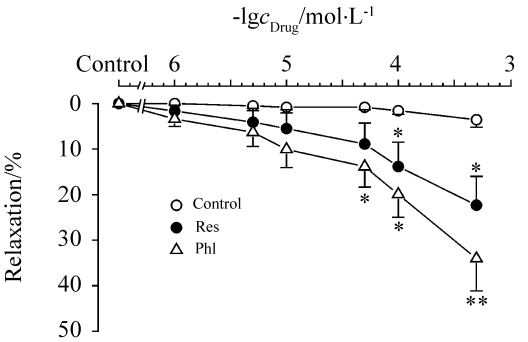

Fig 1. Relaxant effects of resveratrol (Res) and phloretin (Phl) on endothelium-intact rabbit aortic strips precontracted with 40 mmol · L^{-1} KCl. $\bar{x} \pm s$, $n = 8$. $^*P < 0.05$, $^{**}P < 0.01$, compared with control.

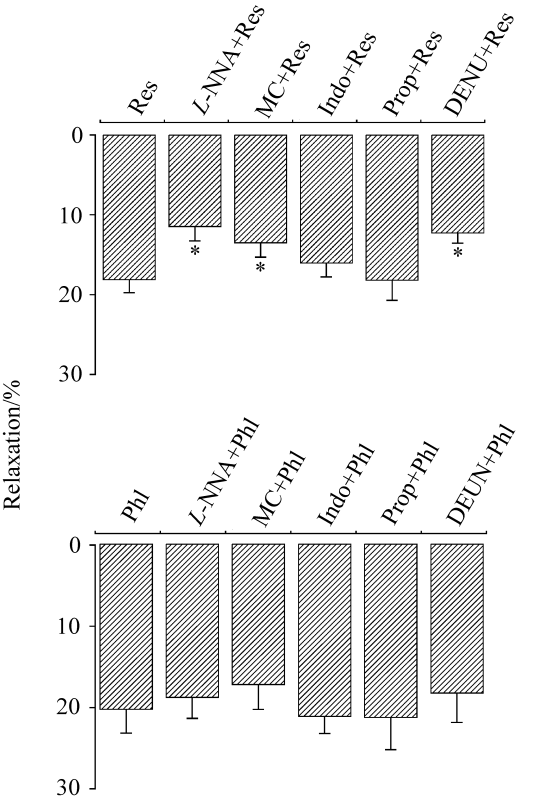

Fig 2. Changes in relaxation induced by resveratrol (250 μmol·L^{-1}) and phloretin (250 μmol·L^{-1}) after preincubation with *L*-NNA, methylthioninium chloride (MC), endothelium removal (DENU), indometacin (Indo) or propranolol (Prop) for 15 min. Data are expressed as relaxing percentage of contraction induced by KCl. $x \pm s$, $n = 6 - 10$. $^*P < 0.05$, compared with corresponding Res or Phl alone.

L^{-1}) in rabbit aortic strips (all $P<0.05$, $n=10$) but did not affect the relaxation induced by 250 μmol·L^{-1} phloretin ($P>0.05$, $n=10$). Incubation with indometacin (10 μmol·L^{-1}) or propranolol (10 μmol·L^{-1}) did not affect relaxation induced by resveratrol and phloretin (all $P>0.05$, $n=6$).

2.3 Effects of resveratrol and phloretin on contractile responses induced by NE and $CaCl_2$

In calcium-free (0.1 mmol·L^{-1} EGTA) Krebs solution, 1 μmol·L^{-1} NE caused a transient contraction. As soon as such contraction reached a plateau, 10 mmol·L^{-1} $CaCl_2$ was rapidly added to the bath and another significant contractile response occurred. Resveratrol (250 μmol·L^{-1}) and phloretin (250 μmol·L^{-1}) reduced the contraction induced by NE in Ca^{2+}-free solution significantly, but did not affect the second contraction caused by $CaCl_2$ (all $P>0.05$, Tab 1).

Tab 1. Inhibitory effects of resveratrol 250 μmol·L^{-1} and phloretin 250 μmol·L^{-1} on contractile responses induced by 1 μmol·L^{-1} NE and 10 mmol·L^{-1} $CaCl_2$

Group	n	Contraction induced by NE/g	Contraction induced by $CaCl_2$/g
Control	11	1.2 ± 0.1	1.5 ± 0.1
Res	11	0.8 ± 0.1*	1.8 ± 0.3
Control	6	0.7 ± 1.5	1.5 ± 4.1
Phl	6	0.4 ± 0.0*	1.5 ± 0.1

$\bar{x} \pm s$. *$P<0.05$, compared with corresponding control.

2.4 Effects of resveratrol and phloretin on NE concentration-dependent contractile responses

Resveratrol (100 and 500 μmol·L^{-1}) and phloretin (100 and 500 μmol·L^{-1}) significantly inhibited NE-induced contraction and made NE contractive curves shifted to the right in a concentration-dependent manner (Fig 3). The pD_2' values of resveratrol and phloretin were 2.89 and 3.23 respectively.

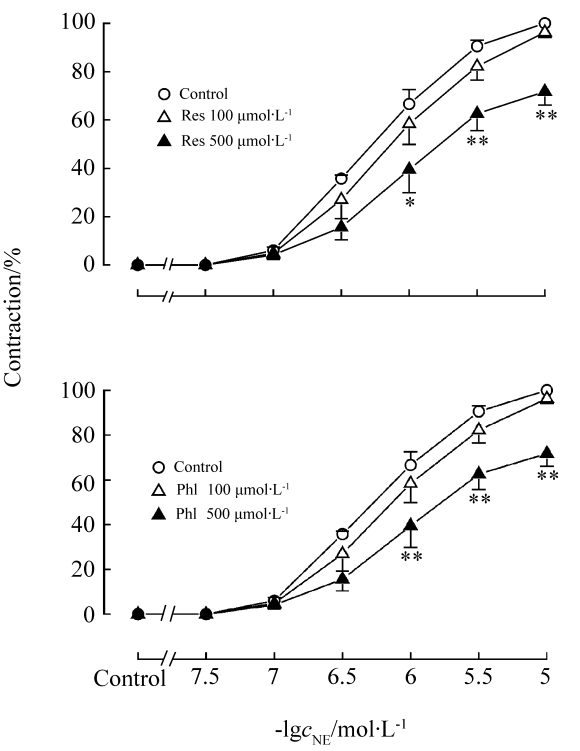

Fig 3. Effects of resveratrol and phloretin on NE induced contraction of rabbit aorta. Maximal contraction induced by NE in control was taken as 100%. $\bar{x} \pm s$, $n=7-9$. *$P<0.05$, **$P<0.01$, compared with control.

2.5 Effects of resveratrol and phloretin on KCl concentration-dependent contractile responses

The KCl concentration-dependent contraction curves were shifted to the right in a concentration-dependent manner after incubation with resveratrol (50, 100 and 500 μmol·L^{-1}) and phloretin (50, 100 and 500 μmol·L^{-1}), and the maximal contraction induced by KCl was reduced significantly (Fig 4). The pD_2' values of resveratrol and phloretin were 3.34 and 3.52 respectively.

2.6 Effect of resveratrol and phloretin on calcium concentration-dependent contraction

Pretreatment with resveratrol (250 μmol·L^{-1}) and phloretin (100 μmol·L^{-1}) led to a reduction in the contractile response to $CaCl_2$ and shifted the concentration-response curve of $CaCl_2$ to the right in high K^+ depolarization medium (Fig 5). The pD_2' values of resveratrol

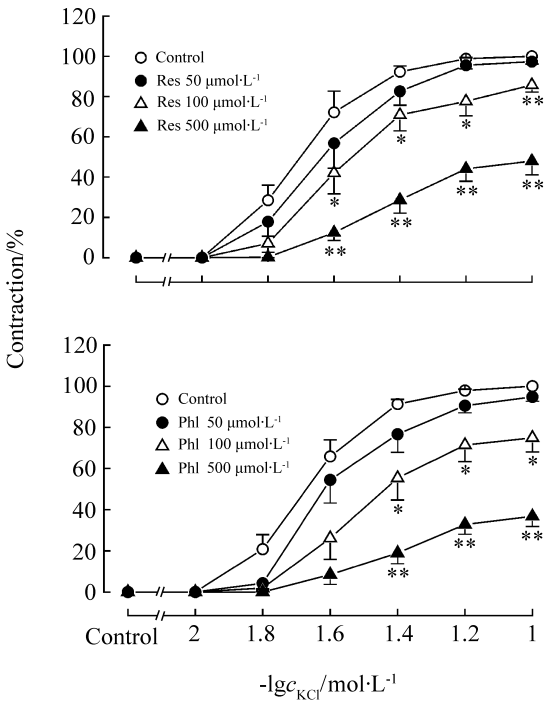

Fig 4. Effects of resveratrol and phloretin on KCl induced contraction of rabbit aorta. Maximal contraction induced by KCl in control was taken as 100%. $\bar{x} \pm s$, n = 7 – 8. $^{*}P<0.05$, $^{**}P<0.01$, compared with control.

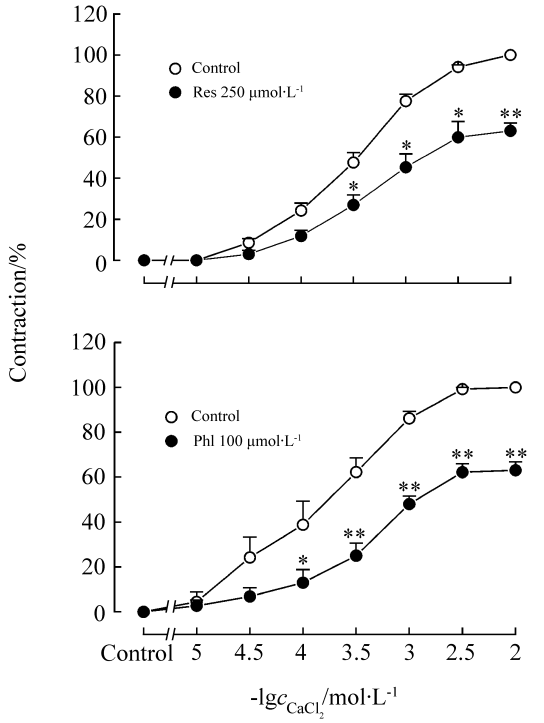

Fig 5. Effects of resveratrol and phloretin on calcium concentration-dependent contraction of rabbit aorta in the presence of 40 mmol·L^{-1} KCl. Maximal contraction induced by calcium in control was taken as 100%. $\bar{x} \pm s$, n = 5. $^{*}P<0.05$, $^{**}P<0.01$, compared with control.

and phloretin were 3.37 and 3.77 respectively.

3 DISCUSSION

The vasorelaxant effects of resveratrol and phloretin have been documented in several studies[3,6,7], but the mechanisms involved are uncertain yet. We found that resveratrol and phloretin could produce acute relaxation, which was concentration-dependent and in a similar way as 17-β-estradiol did[2,8]. Resveratrol induced greater relaxation in rabbit aorta with endothelium than that without. *L*-NNA, an inhibitor of NO synthesis, decreased the relaxation caused by resveratrol. MC, an inhibitor of cGMP synthesis also reduced the relaxation induced by resveratrol. Our results suggest that the relaxation of the rabbit aorta caused by resveratrol is dependent on NO and on cGMP, but the effect of phloretin is independent on the endothelium. These conclusions are supported by other *in vitro* experiments[5,6,9].

In our experiments, indometacin did not affect relaxation induced by resveratrol and phloretin in the intact aorta. Propranolol, an antagonist of β-adrenergic 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 resveratrol and phloretin.

Potential-dependent calcium channels (PDC) are activated by depolarization of the plasma membrane when the extracellular K^+ concentration is increased. In our experiment, incubation with resveratrol and phloretin not only shifted the KCl concentration-dependent contraction curves to the right in normal Krebs solution but also made the calcium concentration-dependent contraction curves move to the right in high K^+ depolarization medium, and inhibited KCl and calcium concentration-dependent contractile responses in a noncompetitive manner. These results support the idea that resveratrol and phloretin have a calcium-antagonistic property and can inhibit Ca^{2+} influx through PDC and

these are similar to estrogen and progesterone[2,10].

NE can activate ROC in the cellular membrane of vascular smooth muscle and increase calcium influx, while it also activates G proteins and phospholipase C to produce inositol trisphosphate (IP_3) which causes calcium release from endoplasmic reticulum[11,12]. In our study, resveratrol and phloretin shifted the NE concentration-dependent curves to the right in a noncompetitive manner. Additionally, in Ca^{2+}-free (0.1 mmol · L^{-1} EGTA) Krebs solution, stimulation of aortic strips with NE to induce Ca^{2+} release from intracellular stores caused one transient contraction, the other contraction appeared after addition of $CaCl_2$ to increase the Ca^{2+} influx through ROC. Resveratrol and phloretin significantly attenuated NE-induced contraction but did not affect $CaCl_2$-induced contraction. Therefore we may conclude that it is the inhibition of IP_3-induced Ca^{2+} release but not Ca^{2+} influx via ROC that is involved in the vasorelaxation caused by resveratrol and phloretin.

In summary, we have shown that resveratrol and phloretin have a direct relaxant effect on rabbit isolated aortic artery. The mechanisms involve the inhibitions of Ca^{2+} influx through PDC and Ca^{2+} release from intracellular stores induced by NE. The relaxing response of resveratrol also partly involves NO and cGMP, but of phloretin is not related to endothelium. Resveratrol and phloretin may play a role in the regulation of aortic vessel tone, and this may contribute to the vasoprotection of phytoestrogens.

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植物雌激素白藜芦醇和根皮素对家兔离体主动脉收缩反应的影响

李红芳[1]，汪龙德[2]，田治峰[3]，李　伟[1]，郑天珍[1]
（兰州大学基础医学院 1. 生理学教研室，3. 医学机能实验室，甘肃 兰州　730000；
2. 甘肃中医学院附属医院，甘肃 兰州　730020）

摘要：目的　探讨植物雌激素白藜芦醇和根皮素对离体主动脉血管收缩的舒张作用特点是否同雌激素以及有关的作用机制。**方法**　将家兔离体主动脉平滑肌条置于灌流肌槽中，记录其等长张力变化。**结果**　白藜芦醇和根皮素可明显抑制离体血管对去甲肾上腺素（NE）、KCl 和 $CaCl_2$ 的浓度依赖性收缩反应，使其量效曲线明显右移，pD_2' 值分别为 2.89，3.34，3.37和3.23，3.52，3.77；对 KCl 预收缩血管条具有浓度依赖性的舒张效应。去除血管内皮、N^{ω}-*L*-硝基精氨酸（*L*-NNA）或亚甲蓝对白藜芦醇舒张血管作用具有明显的抑制作用，但对根皮素诱发的血管舒张无明显影响。吲哚美辛和普萘洛尔温育后，对二者的舒血管作用均无明显影响。在无钙 Krebs 液（含 0.01 mmol · L^{-1} EGTA）中，白藜芦醇和根皮素可抑制 NE 诱发的由肌细胞内钙释放引起的Ⅰ相收缩，但不影响 $CaCl_2$ 诱发的由肌细胞外钙内流引起的Ⅱ相收缩。**结论**　白藜芦醇和根皮素对离体主动脉血管的舒张作用可能与其抑制钙离子内流及细胞内钙释放有关；另外白藜芦醇的舒张作用部分与内皮细胞有关，但根皮素的舒血管作用与内皮细胞无关。

关键词：植物雌激素类；白藜芦醇；根皮素；肌，平滑，血管；主动脉，胸；血管舒张；内皮，血管

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