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A Study of Mechanisms Involved in Vasodilatation Induced by Resveratrol in Isolated Porcine Coronary Artery

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Summary

The present study was designed to investigate the acute relaxing effect of phytoestrogen resveratrol on isolated porcine coronary arteries and to determine the mechanisms underlying its vasodilatation. Rings of porcine coronary arteries were suspended in organ baths containing Krebs-Henseleit solution, and then isometric tension was measured. Resveratrol concentration-dependently relaxed arterial rings precontracted with 30 mM KCl. The IC_{50} value of resveratrol was 38.67±3.21 μM. Incubation with N^{ω}-L-nitro-arginine (L-NNA), endothelium removal or the presence of a potent inhibitor of protein tyrosine phosphatase sodium orthovanadate partly decreased the relaxation induced by resveratrol. However, the relaxation induced by resveratrol was unaffected by the estrogen receptor antagonist tamoxifen, the inhibitor of prostanoid synthesis indomethacin, the antagonist of β-adrenoceptors propranolol or the protein synthesis inhibitor, cycloheximide. In addition, resveratrol significantly decreased the contractile responses of 5-HT, KCl and $CaCl_2$, and shifted their cumulative concentration-response curves to the right. These results suggest that the mechanisms of vasorelaxation induced by resveratrol are heterogeneous, two mechanisms participating partially in the relaxation of porcine coronary artery were detected in the study, one being the nitric oxide released from the endothelium, the other causing inhibition of Ca^{2+} influx, but estrogen receptors were not involved in resveratrol-induced relaxation.

Key words

Resveratrol • Coronary artery • Calcium channel • Vascular endothelium • Nitric oxide

Introduction

Epidemiological data suggest a reduction in the incidence of coronary heart disease in humans who have a high intake of phytoestrogens (Adlercreutz *et al.* 1992, Clarkson and Anthony 1998, Figtree *et al.* 2000). Resveratrol, a phytoestrogen, is naturally occurring phenol compound, which is abundantly found in grape skins and in wines. The similarity in structure between resveratrol and the synthetic estrogen diethylstilbestrol (DES; 4,4′-dihydroxy-trans-α, β-diethylstilbene) made resveratrol to exhibit variable degrees of estrogen

receptor agonism (Gehm *et al.* 1997) and estrogenic activity (Lobo 1998, Orallo *et al.* 2002). Some papers demonstrated that resveratrol relaxes isolated vascular arteries (Fitzpatrick *et al.* 1993, Jager and Nguyen-Dong. 1999, Naderali *et al.* 2000, 2001), has a potent anti-inflammatory and anti-oxidant effect, and can also improve ventricular function and decrease lactic dehydrogenase release after ischemia in rats (Hale and Kloner 2001). Thus, its biologic effect is very useful for medicine and nutrition, and it is proposed to account in part for the protective effect of red wine on the cardiovascular system (Bruder *et al.* 2001, Haider *et al.* 2002). Despite the increasing interest in the effects of resveratrol on the cardiovascular system, there is some discrepancy in the mechanisms involved in its effect and which are not completely understood yet. It is also not known whether it shares the same vasodilator properties of estrogen. Therefore, the purpose of this study was to investigate the acute relaxing effect of phytoestrogen resveratrol on isolated porcine coronary arteries and its possible mechanisms, such as the roles of nitric oxide, prostaglandins, β-adrenoceptors, calcium influx and estrogen receptor in resveratrol-induced vasorelaxation.

Methods

Tissue preparation

Fresh porcine hearts of either sex were obtained from a local abattoir in cold, modified Krebs-Henseleit (K-H) solution of the following composition (in mM): NaCl 120, KCl 4.76, NaH_2PO_4 1.18, $MgSO_2$ 1.18, $NaHCO_3$ 25, $CaCl_2$ 1.25, and glucose 5.5. The left anterior descending (LAD) coronary was excised rapidly and placed into K-H buffer solution bubbled with 95 % O_2 and 5 % CO_2, then carefully cleaned of connective tissue and blood. The proximal LAD (3-5 cm from the portal) was cut into 4 mm ring segments. Ring samples were then suspended horizontally on two stainless steel hooks in a tissue chamber containing oxygenated (95 % O_2 and 5 % CO_2) K-H solution at 37 °C. Isometric tension generated by coronary smooth muscle was measured using a force transducer (JH-2, Beijing, China) and recorded with BL-420 Experimental System of Biological Function (TME, China) through IBM computer. Resting tension was set to 1.5 g. After 90 min of equilibration, the rings were activated with KCl (30 mM) to check their integrity.

In some arterial rings, the endothelium was removed by gentle rubbing the intimal surface of the vessel with a cotton swab. Endothelial integrity was assessed pharmacologically by the ability of bradykinin (1 μM) to produce relaxation of tissues precontracted with $PGF_{2\alpha}$ (10 μM) (Fig. 1).

Drugs

Resveratrol, N^{ω}-L-nitro-arginine (L-NNA), bradykinin, tamoxifen, indomethacin, sodium orthovanadate, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), 5-hydroxy-tryptamine (5-HT), propranolol and cycloheximide were obtained from Sigma Chemical (St. Louis, Mo., USA). Resveratrol and tamoxifen were dissolved in dimethylsulfoxide (DMSO). The final concentration of DMSO in the bath never surpassed 0.5 %, and it had no effect on the tone of isolated coronary arteries. Indomethacin was dissolved in a Na_2CO_3 solution at pH 7.4. The stock solutions of remaining drugs were dissolved in distilled water.

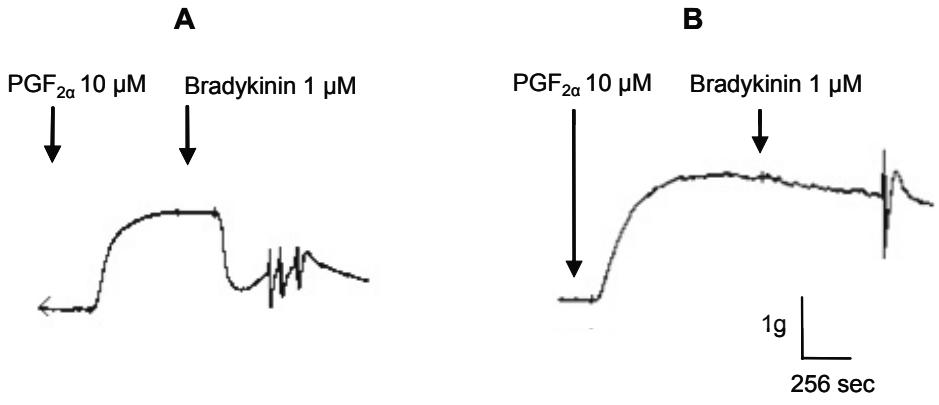

Fig 1. The actual tracings to indicate the influence of endothelial integrity assessed by the ability of bradykinin (1 μM) to produce relaxation of the preparation precontracted with $PGF_{2\alpha}$ (10 μM). A: Endothelium-intact; B: Endothelium-denuded.

Relaxing effect of resveratrol on precontracted coronary arteries

Coronary arterial rings with or without endothelium were precontracted with 30 mM KCl. When the contractile response had reached a stable plateau (approximately 15-20 min), increasing concentrations of resveratrol (1-100 μM) were added in 10-min intervals. The response to each concentration of resveratrol was measured. Simultaneous time-matched DMSO control was constructed using an equivalent volume of the solvent as that used for dissolving resveratrol.

Comparative relaxing effects of resveratrol in the presence of a variety of inhibitors

In some experiments, rings with endothelium were once again treated with 30 mM KCl and the responses to resveratrol (1-100 μM) were measured after 20 min preincubation with one of the following specific inhibitors: 100 μM L-NNA, 10 μM tamoxifen, 10 μM sodium orthovanadate, 10 μM indomethacin, 10 μM propranolol or 10 μM cycloheximide, respectively.

Effects of resveratrol on 5-HT, KCl or calcium concentration-response curves in endothelium-denuded coronary rings

Rings were stabilized at 1.5 g resting tension for 90 min in K-H solution and the concentration-response curve to 5-HT (0.01-10 μM) was then obtained. After washout of 5-HT, the experiment was repeated in the presence of resveratrol (1 μM or 10 μM).

The concentration-response curve to KCl (10-100 mM) was observed in the absence or presence of resveratrol (1 μM, 10 μM or 50 μM).

Coronary arterial rings were incubated in calcium-free solution containing 0.01 mM EGTA for 60 min. The calcium concentration-dependent contraction curve was then measured in K^+ depolarization medium (80 mM KCl). Rings were readjusted in normal Krebs-Henseleit solution for 10 min and then incubated in calcium-free solution for a further 40 min. Subsequently, the rings were incubated with resveratrol (30 μM) for 20 min and the calcium concentration-dependent contraction curve was then repeated.

Data analysis

All results are expressed as means ± S.E.M., n refers to the number of hearts used in the study. Relaxation was expressed as the percentage relaxation of the contraction induced by KCl (30 mM). In experiments involving the concentration-response curves, the results were expressed as the percentage of the maximal contractile response induced by 100 mM KCl, $10^{-5.5}$ M 5-HT and 10^{-2} M $CaCl_2$ in the controls respectively. Statistical analysis was performed using Student's t-test and analysis of variance (ANOVA). Each group was compared with the solvent control. $P<0.05$ value was considered significant.

Results

Relaxing effect of resveratrol on KCl-precontracted coronary arteries

In KCl (30 mM) precontracted endothelium-intact coronary rings, phytoestrogen resveratrol induced a concentration-dependent relaxation response ($r=0.97$, $p<0.01$, $n=18$) as compared to time-matched solvent control (Fig. 2). The IC_{50} value of resveratrol was 38.67±3.21 μM.

Effects of L-NNA, endothelium removal on the resveratrol-induced vasorelaxation

Incubation with the inhibitor of NO synthesis, L-NNA (100 μM), or endothelium removal partially but significantly reduced the concentration-dependent vasorelaxation induced by resveratrol in porcine coronary rings (all $p<0.05$, $n=8$-15) (Fig. 2). The maximal relaxation was reduced from 94.10±2.41 % to 72.812±6.91 % ($p<0.05$, $n=8$) and 67.88±9.51 % ($p<0.05$, $n=8$), respectively.

Effects of a variety of inhibitors on the resveratrol-induced vasorelaxation

Incubation with tamoxifen (10 μM), an estrogen-receptor antagonist, or the inhibitor of prostanoid synthesis, indomethacin (10 μM) did not inhibit the concentration-dependent vasorelaxation induced by resveratrol in porcine coronary rings with endothelium (all $p>0.05$, $n=6$-15, Fig 3A). In 30 mM KCl- precontracted coronary rings, pretreatment with sodium orthovanadate (10 μM), a potent inhibitor of protein tyrosine phosphatase partly inhibited the concentration-dependent vasorelaxation caused by resveratrol, the maximal relaxation was reduced from 94.10±2.41 % to 73.13±3.46 % ($p<0.05$, $n=8$, Fig. 3A). Propranolol (10 μM) or cycloheximide (10 μM) was added to the baths 20 min prior to addition of resveratrol; it was not possible to reverse the relaxant effect of resveratrol on the precontracted porcine coronary arterial rings (Fig 3B, $n=6$).

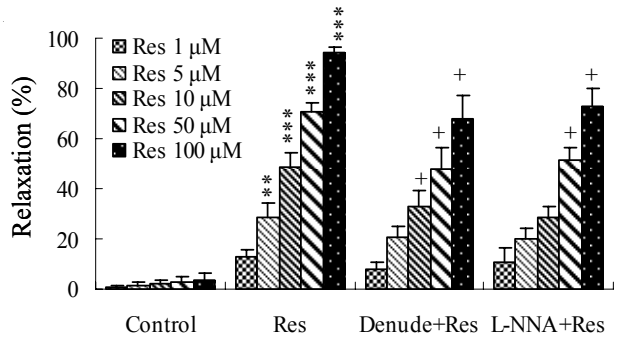

Fig 2. Relaxant effects of resveratrol (Res, 1-100 μM) on endothelium-intact, endothelium-denuded (Denude), or L-NNA incubated (L-NNA) porcine coronary arterial rings precontracted with 30 mM KCl. Data are expressed as the percentage of relaxation in 30 mM KCl-precontracted arterial rings (mean ± S.E.M.). Control indicates a time-matched equivalent volume of solvent. n=8-15. ***P*<0.01, ****P*<0.001 *vs* control; $^{+}$*P*<0.05 *vs* Res.

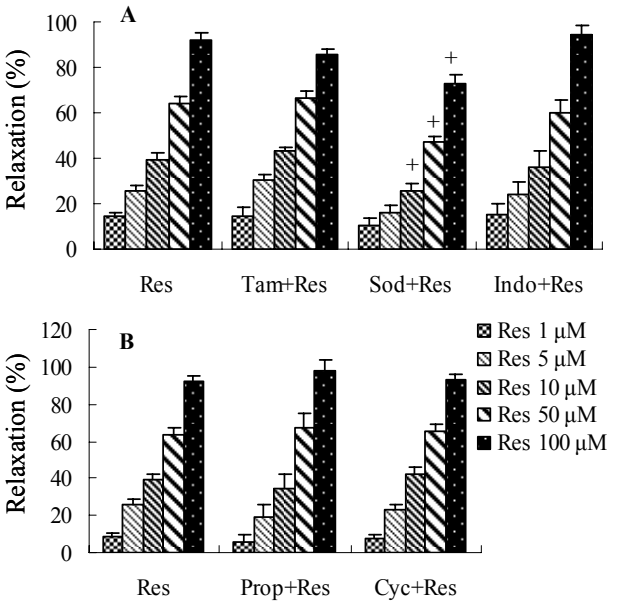

Fig 3. (A) The effect of tamoxifen (Tam), sodium orthovanadate (Sod) or indomethacin (Indo) on the resveratrol-induced relaxation in porcine coronary rings with endothelium. $^{+}$*P*<0.05 *vs* Res group. (B) The effect of incubation in Propranolol (Prop) or cycloheximide (Cyc) on resveratrol-induced relaxation in coronary rings with endothelium. n=6-15.

The concentration-response curves for KCl (10-100 mM) were shifted to the right in a concentration-dependent manner after incubation with resveratrol (1 μM, 10 μM or 50 μM), and the maximal contraction for KCl was reduced to 98.85±5.70 %, 77.26±8.71 % ($p<0.05$ vs control, n=8) and 41.80±7.69 % ($p<0.001$ vs control, n=8), respectively (Fig 4). PD_2 values of KCl in control and after incubation with resveratrol (1 μM, 10 μM or 50 μM) were 1.82±0.10, 1.68±0.10, 1.42±0.10 ($p<0.01$ vs control, n=8) and 1.07±0.09 ($p<0.001$ vs control, n=8), respectively.

Inhibitions by resveratrol of the concentration-response curve for calcium

The concentration-response curve for calcium in high K^+ depolarization medium was shifted to the right after incubation with resveratrol (30 μM) in coronary rings without endothelium compared with the controls. The maximal contraction of $CaCl_2$ was reduced to 46.07±6.62 % ($p<0.001$ vs control, n=6) (Fig. 5A). PD_2 values of $CaCl_2$ for control and after incubation with 30 μM resveratrol were 2.54±0.06 and 1.96±0.07 ($p<0.001$ vs control, n=6) respectively.

Inhibitions by resveratrol of the 5-HT-induced contractile response

A concentration-dependent contractile response could be induced by 5-HT (0.01-10 μM) in isolated porcine coronary arteries. However, after incubation with resveratrol (1 μM or 10 μM), the concentration-response curve for 5-HT was shifted to the right in a dose-dependent manner, and the maximal contraction of 5-HT was reduced to 73.08±7.02 % ($p<0.01$ vs control, n=8) and 33.97±7.86 % ($p<0.001$ vs control, n=8), respectively (Fig. 5B). PD_2 values of 5-HT in control and after incubation with resveratrol (1 μM or 10 μM) were 5.67±0.02, 5.56±0.11 and 4.99±0.14 ($p<0.01$ vs control, n=8), respectively.

Discussion

We have demonstrated that phytoestrogen resveratrol (1-100 μM) induces a concentration-dependent relaxation in precontracted porcine coronary rings. El-Mowafy (2002) reported that resveratrol could activate membrane-bound guanylyl cyclase in sheep coronary arterial smooth muscle, which was dependent on endothelium and nitric oxide (NO). This novel signaling mechanism supports coronary protection. In the present experiment, the relaxation of porcine coronary arteries with endothelium was greater than that without endothelium. L-NNA, an inhibitor of NO synthesis, also significantly decreased the relaxation caused by resveratrol. These results suggest that the relaxation of the porcine coronary arteries caused by resveratrol is partly related to NO and the endothelium in part. Our results are consistent with previous findings of *in vitro* experiments (Fitzpatrick *et al.* 1993, Naderali *et al.* 2001, El-Mowafy 2002).

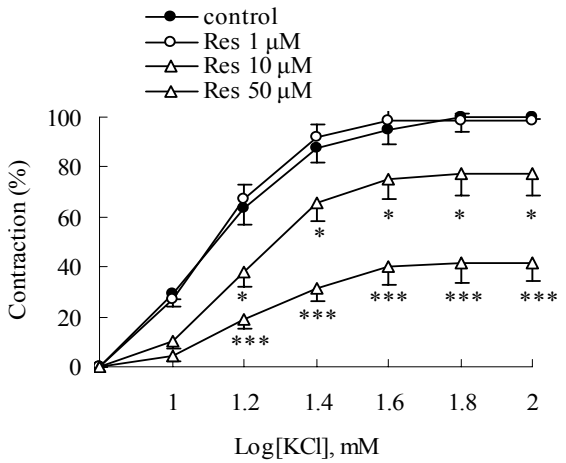

Fig 4. Inhibitions by resveratrol (Res, 1, 10 or 50 μM) of the KCl -induced contractile response in porcine coronary arteries without endothelium. Data are expressed as the percentage of the maximal contractile response induced by KCl in controls (means ± S.E.M.). n=8. **P*<0.05, ****P*<0.001 *vs* control.

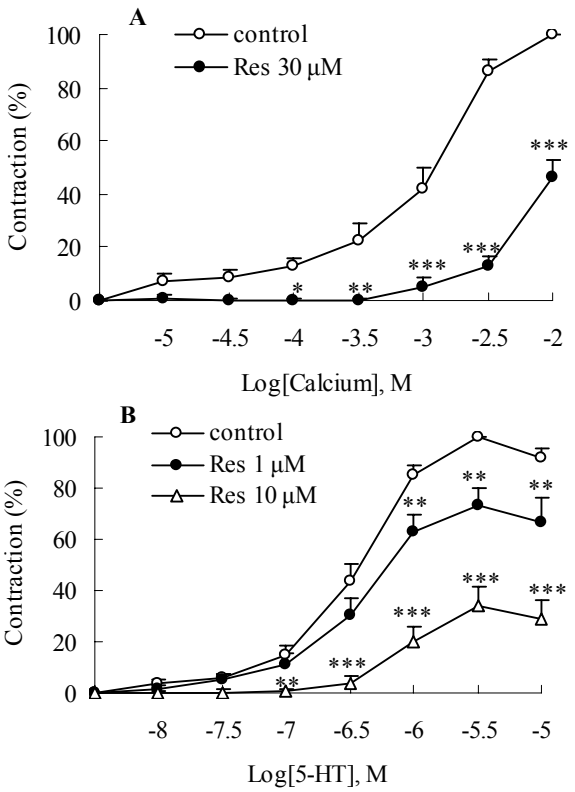

Fig 5. (A) Inhibitions by resveratrol (Res, 30 μM) of the concentration-response curve for calcium in porcine coronary arteries without endothelium. Data are expressed as the percentage of the maximal contractile response induced by calcium in controls (mean ± S.E.M.). (B) Inhibitions by resveratrol (Res, 1 or 10 μM) of the 5-HT-induced contractile response in porcine coronary arteries without endothelium. Data are expressed as percentage of the maximal contractile response induced by 5-HT in controls (mean ± S.E.M.). n=6-8. **P*<0.05, ***P*<0.01, ****P*<0.001 *vs* control.

Some evidence support the cardioprotective role of NO in this connection (Flogel *et al.* 1999). For example, an NO donor or a precursor for NO synthesis like L-arginine has been found to ameliorate myocardial ischemic-reperfusion injury (Hattori *et al.* 2002). Resveratrol, a polyphenol phytoalexin (trans-3,4′,5-trihydroxystilbene), has been found to protect the heart from ischemic-reperfusion injury (Hattori *et al.* 2002), and to enhance the constitutive NO formation in some tissues including the kidney and the heart. This compound thus possesses diverse biochemical and physiological actions, which include estrogenic, antiplatelet and anti-inflammatory properties (Hattori *et al.* 2002). Recently, resveratrol was found to protect the kidney, heart, and brains from ischemic-reperfusion injury (Ray *et al.* 1999, Hattori *et al.* 2002). We speculate that the protective effect of resveratrol on cardiovascular system may partially be related to its NO-dependent vasorelaxation.

Evidence has been presented to suggest that enhanced tyrosine phosphorylation participates in the mechanisms that regulate the contraction of smooth muscle (Kitazono *et al.* 1998). For instance, Suenaga and Kamata (2002) recently reported that sodium orthovanadate, a tyrosine phosphatase inhibitor, markedly enhances the high K^+-induced contractile responses. In the present study, it was found that sodium orthovanadate partly inhibited resveratrol-induced relaxation, which may be related to its ability of decreasing eNOS activity through tyrosine phosphorylation-dependent mechanisms in the systemic circulation (Garcia-Cardena *et al.* 1996) and reducing NO-mediated pulmonary vasodilatation and endothelium-dependent relaxation (Zerrouk *et al.* 1999, Huang *et al.* 2002). These findings are consistent with the observation that the relaxant effect of resveratrol was partly NO-dependent.

Indomethacin inhibits the synthesis of prostaglandins and markedly decreases the transient relaxation induced by arachidonic acid in rabbit coronary arteries. However, in the present study, indomethacin did not affect resveratrol-induced relaxation in endothelium-intact coronary arteries. Propranolol, an antagonist of the β-adrenoceptors, also did not affect vasorelaxation. These results indicate that the release of vasodilator prostanoids and β-adrenoceptors are not involved in resveratrol-induced relaxation in isolated coronary arteries.

Estrogens, including phytoestrogens, act *via* the estrogen receptor, a member of nuclear receptor superfamily. Both the "classical" estrogen receptor ERα and the novel ERβ have been detected in arteries and in

cultured vascular smooth muscle cells of cynomolgus monkeys as well as in rat arteries (Makela *et al.* 1999). Estrogen binding to the receptor activates the transcription of estrogen-responsive target genes. Gehm *et al.* (1997) has demonstrated that resveratrol is a phytoestrogen and exhibits variable degrees of estrogen receptor agonism in different test systems. In addition, because resveratrol is structurally or functionally similar to estrogen, which is a lipid-soluble substance and its molecular weight is not large, it can easily enter the cytoplasm through the cellular membrane to affect the expression of some genes. However, in the present experiments, the lack of inhibition of coronary artery relaxations induced by resveratrol after incubation with the estrogen receptor antagonist tamoxifen or the protein synthesis inhibitor cycloheximide suggests that estrogen receptor probably does not play a role in the acute vasorelaxant effects of resveratrol in isolated porcine coronary arteries. Our data support the concept that the acute vasorelaxant effects caused by resveratrol are not mediated by the classical estrogen receptor but are independent of gene-mediated events.

Voltage-dependent calcium channels (VDCCs) are activated by depolarization of the plasma membrane when the extracellular K^+ concentration is increased. In our experiments, incubation with resveratrol not only shifted the concentration-response curve for KCl to the right in normal K-H solution but also moved the concentration-response curve for calcium to the right in high K^+ depolarization medium, and inhibited KCl concentration-dependent contractile response in a parallel manner. These results are consistent with the effect of 17-β estradiol on a large elastic aorta as we reported previously (Li *et al.* 2002) and are also supported by another study (Nevala *et al.* 1998). These results suggest that resveratrol may have Ca^{2+} antagonistic properties and can inhibit extracellular Ca^{2+} influx through VDCCs, which are similar to those of 17-β estradiol.

In addition, 5-HT is released from activated platelets and has an obvious vasoconstricting effect which can be markedly reduced in the absence of extracellular Ca^{2+} or by application of verapamil (an L-type Ca^{2+} channel blocker), so that the contraction induced by 5-HT involves voltage-dependent Ca^{2+} channels and transplasmalemmal Ca^{2+} entry (Tasaki *et al.* 2003). In our experiment we found that resveratrol, just as verapamil (Tasaki *et al.* 2003), could markedly inhibit the contractile response to 5-HT, and shift its concentration-response curve to the right in a parallel manner. These data also support the Ca^{2+} antagonistic properties of resveratrol. Certainly, the other mechanisms such as the blockade of 5-HT receptors or some hyperpolarizing factor may probably be involved in the inhibitory effect of resveratrol on the 5-HT-induced contraction, which will need further investigation.

In conclusion, the mechanisms of vasorelaxation induced by resveratrol are heterogeneous, two mechanisms participating partially in the relaxation of porcine coronary artery were detected in the present study. One concerns the nitric oxide released from the endothelium, the other is the inhibition of Ca^{2+} influx, but the estrogen receptors were not involved in the resveratrol-induced relaxation. Therefore, resveratrol has vasodilatory effects similar to those of 17-β estradiol (Li *et al.* 2002), and may be clinically useful as a safer substitute for feminizing estrogens in preventing cardiovascular diseases.

Acknowledgements

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Reprint requests

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