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Differential mechanisms involved in effects of genistein and 17-β-estradiol on porcine coronary arteries

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The purpose of this work was to examine the differential mechanisms involved in relaxation induced by genistein and 17-β-estradiol in isolated porcine coronary arteries. Similar to 17-β-estradiol, genistein could dose-dependently relax 30 mM KCl-precontracted coronary artery rings. The pD_2 values of genistein and 17-β-estradiol were 4.91 ± 0.13 and 4.98 ± 0.12 respectively. Incubation with *N*-L-nitro-arginine (L-NNA), endothelium removal or in the presence of a potent inhibitor of protein tyrosine phosphatase sodium orthovanadate did not affect the relaxation induced by genistein, but could partially reduce the vasorelaxation induced by 17-β-estradiol. The relaxations induced by genistein and 17-β-estradiol were unaffected by the estrogen receptor antagonist tamoxifen, the inhibitor of prostanoid synthesis indomethacin and the protein synthesis inhibitor, cycloheximide. In addition, both of genistein and 17-β-estradiol could decrease the contractile responses of KCl, 5-HT and $CaCl_2$, and shift their cumulative concentration-response curves rightward in a parallel manner. These findings suggest that the relaxant effects induced by genistein and 17-β-estradiol are probably mainly due to inhibition of Ca^{2+} influx through voltage-dependent calcium channels (VDCCs), and are not related to sex hormone receptor and classical genomic activities. Also there is an interesting finding that the relaxing response of 17-β-estradiol is partially endothelium-dependent, but that of genistein is not.

1. Introduction

Genistein, a phytoestrogen, may have estrogenic cardioprotective actions (Barnes 1998). Epidemiological data suggest a reduction in the incidence of coronary heart disease in humans who have a high intake of phytoestrogens (Clarkson and Anthony 1998). Increased plasma levels of the phytoestrogen genistein are suggested as an explanation for the infrequency of hot flashes and menopausal symptoms in women (Adlercreutz et al. 1992; Hertog et al. 1995). The affinity of genistein for the classic estrogen α-receptor present on reproductive organs is less than that of estrogen (Cassidy 1999). However, genistein has a similar affinity as estrogen for the estrogen β-receptor in the vasculture (Walker et al. 2001). Many studies demonstrate that 17-β-estradiol has a direct vasodilatory action *in vitro*, which may partly contribute to cardiovascular protection (Andersen et al. 1999; Li et al. 2002). Recently there is evidence that genistein also can be vasoactive, it potentiates coronary vasoreactivity in macaque monkeys (Honore et al. 1997), relaxes rat mesenteric and rabbit coronary arteries *in vitro* (Honore et al. 1997; Nevala et al. 1998), and also dilates human forearm vasculature *in vivo* (Walker et al. 2001). However, the mechanism involved is not completely understood, it is uncertain whether the vasoreactivities and the signal transduction mechanisms of genistein and 17-β-estradiol are completely the same in arterial smooth muscle, and even less is known about the importance of phosphorylation and dephosphorylation of tyrosyl residues in the vasorelaxation induced by a potent tyrosine kinase inhibitor genistein. The purpose of the present study was to compare the effects of genistein on coronary arterial smooth muscles with those of 17-β-estradiol *in vitro* and, more important, to determine the differential mechanisms involved.

2. Investigations and results

2.1. Relaxing effects of genistein and 17-β-estradiol on KCl-precontracted coronary arteries

In KCl (30 mM) precontracted endothelium-intact coronary arterial rings, genistein or 17-β-estradiol could dose-dependently induce a relaxation response (all $r = 0.94$, $p < 0.01$, $n = 13$) when compared with time-matched solvent controls (Fig. 1A and Fig. 1B). The pD_2 values of genistein and 17-β-estradiol were 4.91 ± 0.13 and 4.98 ± 0.12 respectively.

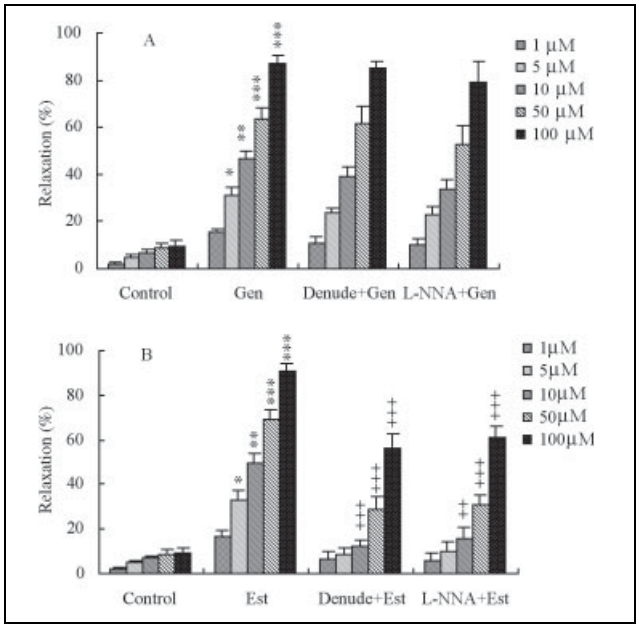

Fig. 1: Relaxant effects of genistein (Gen, 1–100 μM) (A) and 17-β-estradiol (Est, 1–100 μM) (B) on endothelium-intact, endothelium denuded (Denude), or L-NNA incubated (L-NNA) porcine coronary arterial rings precontracted with 30 mM KCl. Data are expressed as percentage relaxation of contraction by 30 mM KCl (mean ± se). Control indicates a time-matched equivalent volume of solvent. n = 7–13. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control; ++ $p < 0.01$, +++ $p < 0.001$ vs Gen (A) or Est (B) group

2.2. *Effects of L-NNA, endothelium removal on dose-dependent vasorelaxation of genistein and 17-β-estradiol in KCl-contracted coronary arterial rings*

Incubation with the inhibitor of NO synthesis, L-NNA (100 μM), or endothelium removal did not affect the dose-dependent vasorelaxation induced by genistein (all $p > 0.05$, n = 7, Fig. 1A), but partially reduced the dose-dependent vasorelaxation induced by 17-β-estradiol in porcine coronary arterial rings ($p < 0.001$, n = 7, Fig. 1B).

2.3. *Effects of a variety of inhibitors on dose-dependent vasorelaxation induced by genistein and 17-β-estradiol in KCl-contracted coronary arteries*

Incubation with tamoxifen (10 μM), an estrogen-receptor antagonist, or the inhibitor of prostanoid synthesis, indomethacin (10 μM) did not inhibit the dose-dependent vasorelaxation induced by genistein and 17-β-estradiol in porcine coronary arterial rings with endothelium (all $p > 0.05$, n = 8–13, Fig. 2A and Fig. 2B). Sodium orthovanadate (10 μM), a potent inhibitor of protein tyrosine phosphatase had no contractile effect on basal tone but significantly increased the magnitude of KCl (30 mM)-induced contraction in all coronary rings ((5.16 ± 0.29) g vs (6.14 ± 0.39) g; $p < 0.01$, n = 12). In 30 mM KCl-precontracted coronary rings, pretreatment with sodium orthovanadate (10 μM) did not affect the relaxation induced by genistein ($p > 0.05$, n = 10, Fig. 2A), but could partly inhibit the dose-dependent vasorelaxation caused by 17-β-estradiol ($p < 0.05$, n = 8, Fig. 2B). Cycloheximide (10 μM) was added to the baths 20 min prior to additions of genistein or 17-β-estradiol, it was not able to reverse the relaxant effects of genistein or 17-β-estradiol in porcine coronary arterial rings precontracted by KCl (Fig. 3A and Fig. 3B, n = 4).

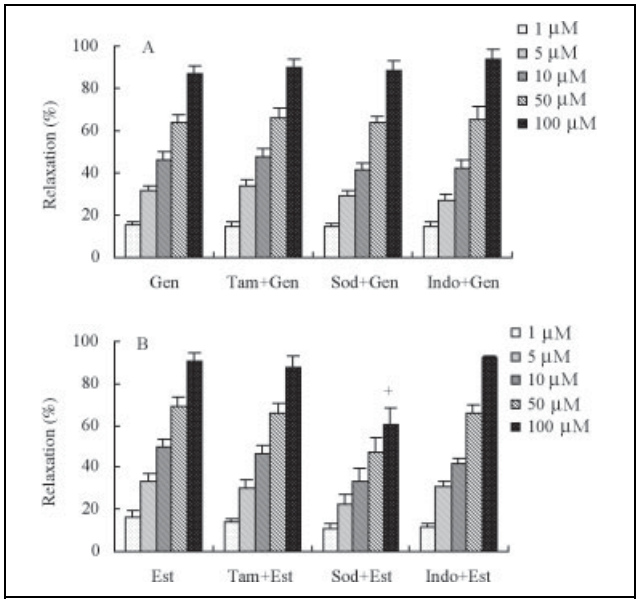

Fig. 2: Effects of incubation in tamoxifen (Tam), sodium orthovanadate (Sod) or indothelium (Indo) on (A) genistein and (B) 17-β-estradiol-induced relaxation in coronary rings with endothelium. n = 8–13. + $p < 0.05$ vs Gen (A) or Est (B) group

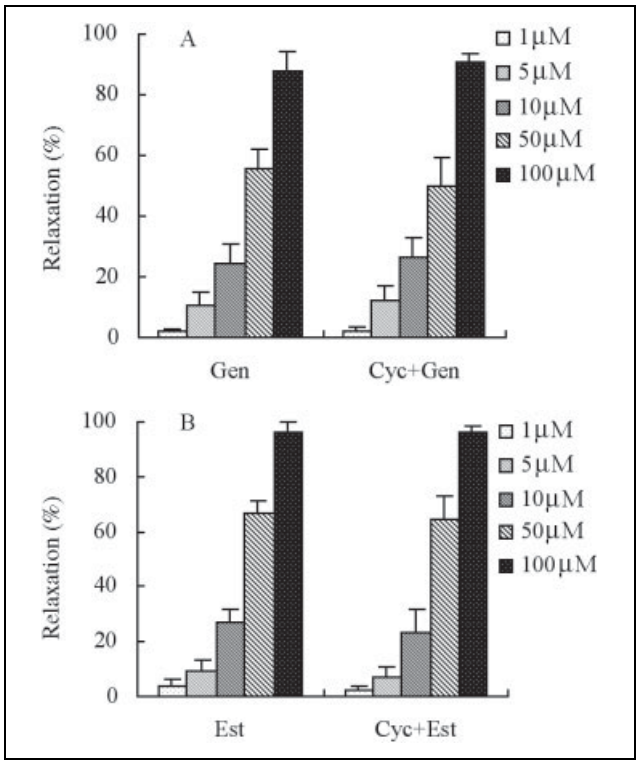

Fig. 3: Effects of incubation in cycloheximide (Cyc) on (A) genistein and (B) 17-β-estradiol-induced relaxation in coronary rings with endothelium. n = 4

2.4. *Inhibition of KCl concentration-dependent contractile responses by genistein and 17-β-estradiol*

The KCl (5–60 mM) concentration-dependent contraction curves were shifted to the right in a dose-dependent manner after incubation with genistein (1 μM, 30 μM or 100 μM) or 17-β-estradiol (1 μM, 30 μM or 100 μM), and maximal contractions were also reduced (Fig. 4A and Fig. 4B). The pD_2 values of KCl in control and after incu-

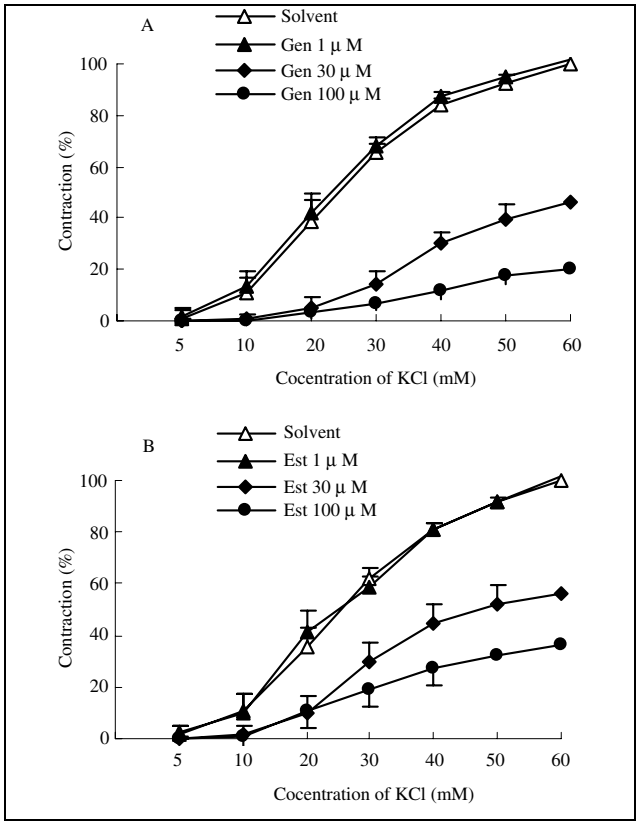

Fig. 4: Effects of genistein (Gen, 1, 30 or 100 μM) (A) and 17-β-estradiol (Est, 1, 30 or 100 μM) (B) on KCl concentration-dependent contraction curves in porcine coronary arteries without endothelium. Data are expressed as percentage of maximal contraction induced by KCl in controls (mean ± se). n = 10

bation with genistein (1 μM, 30 μM or 100 μM) were 1.78 ± 0.25, 1.88 ± 0.27, 0.90 ± 0.15 and 0.47 ± 0.09 ($p < 0.01$ vs control, n = 10), and in control and after incubation with 17-β-estradiol (1 μM, 30 μM or 100 μM) were 1.73 ± 0.24, 1.70 ± 0.25, 1.17 ± 0.13 and 0.92 ± 0.04 ($p < 0.01$ vs control, n = 10) respectively.

2.5. Inhibition of 5-HT concentration-dependent contractile responses by genistein and 17-β-estradiol

The concentration-dependent contractions could be elicited by 5-HT (0.01–10 μM) in isolated coronary arterial rings. However, after incubation with genistein (30 nM or 1 μM) or 17-β-estradiol (30 nM or 1 μM), the 5-HT concentration-dependent contraction curves were shifted to the right in a dose-dependent manner, and maximal contractions were also reduced (Fig. 5A and Fig. 5B). The pD_2 values of 5-HT in control and after incubation with genistein (30 nM or 1 μM) were 6.08 ± 0.13, 5.91 ± 0.14 and 5.20 ± 0.12 ($p < 0.05$ vs control, n = 10), and in control and after incubation with 17-β-estradiol (30 nM or 1 μM) were 6.07 ± 0.19, 5.83 ± 0.18 and 5.22 ± 0.30 ($p < 0.05$ vs control, n = 10) respectively.

2.6. Influence of genistein and 17-β-estradiol on calcium concentration-dependent contraction

The calcium concentration-dependent contraction curves of high K^+ (80 mM) depolarized tissues in Ca^{2+}-free medium were shifted to the right after incubation with genistein (30 μM) or 17-β-estradiol (30 μM) in coronary rings without endothelium compared with controls, the contractile responses of calcium were obviously decreased (Fig. 6A and Fig. 6B). The pD_2 values of $CaCl_2$ for control and after incubation with 30 μM genistein were 2.74 ± 0.23 and 2.20 ± 0.09 ($p < 0.05$ vs control, n = 7), and for control and after incubation with 30 μM 17-β-estradiol were 2.75 ± 0.30 and 1.87 ± 0.15 ($p < 0.01$ vs control, n = 7) respectively.

3. Discussion

The present study has shown that the phytoestrogen genistein can induce significant relaxation of coronary artery rings in a way similar to 17-β-estradiol. The relaxation was dose-dependent. The reports on effects of NO and endothelial cells on vasorelaxation induced by genistein are not consistent (Walker et al. 2001; Ho et al. 2002; Mishra et al. 2000). In the present study, no differences were observed between coronary rings with or without endothelium. Preincubation with L-NNA, an inhibitor of NO synthesis did not affect genistein-induced vasorelaxation. However, the relaxing effect of 17-β-estradiol was partly endothelium-dependent and related to NO, which is supported by others experiments (Donna and Clare 1998; Geary et al. 1998). These results also agree with our previous study in rabbit aortic arteries (Li et al. 2004), and suggest that the relaxation of coronary artery rings caused by genistein is independent on endothelium and NO. Just as Marsh (2000) reported that this is of therapeutic interest, as conceivably genistein might be used in patients with diseased and dysfunctional endothelium.

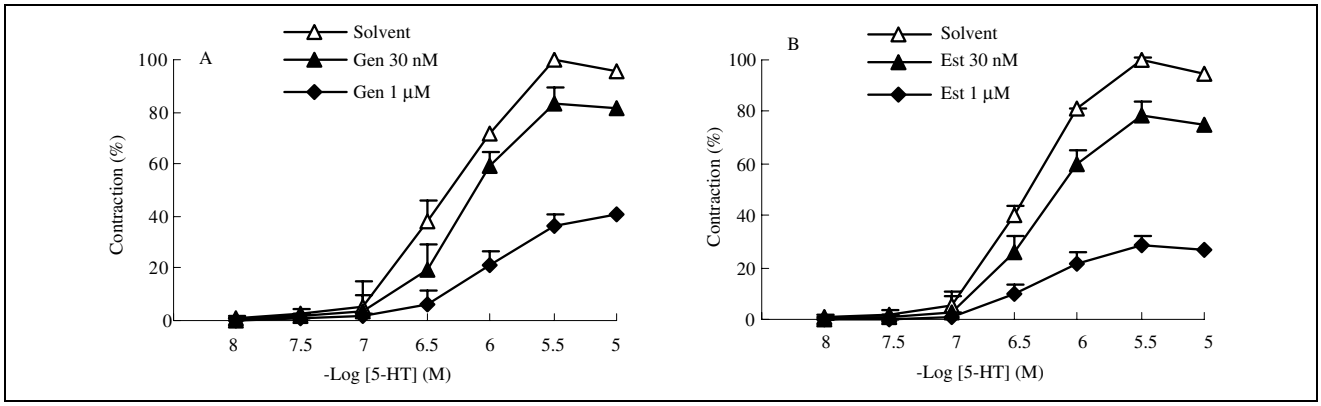

Fig. 5: Effects of genistein (Gen, 30 nM or 1 μM) (A) and 17-β-estradiol (Est, 30 nM or 1 μM) (B) on 5-HT concentration-dependent contraction curves in porcine coronary arteries without endothelium. Data are expressed as percentage of maximal contraction induced by 5-HT in controls (mean ± se). n = 10

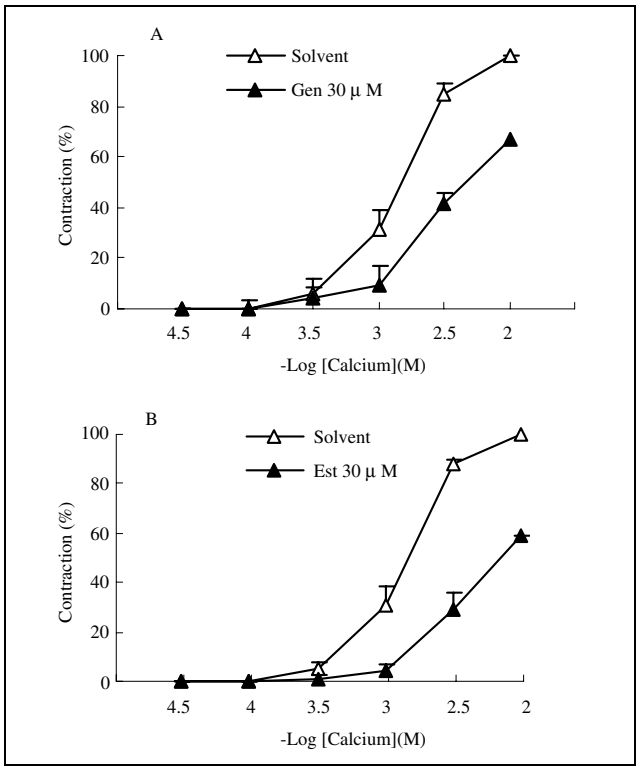

Fig. 6: Effects of genistein (Gen, 30 μM) (A) and 17-β-estradiol (Est, 30 μM) (B) on calcium concentration-dependent contraction curves in porcine coronary arteries without endothelium. Data are expressed as percentage of maximal contraction induced by calcium in controls (mean ± se). n = 7

Genistein, a well-known selective tyrosine kinase inhibitor, just as other tyrosine kinase inhibitors, can suppress arterial contraction responses to noradrenaline and potassium chloride (Masui and Wakabayashi 2000). Evidence (Kitazono et al. 1998) has also been presented to suggest that enhanced tyrosine phosphorylation participates in the mechanisms that regulate the contraction of smooth muscle. For instance, Suenaga and Kamata (2002) reported that sodium orthovanadate, a tyrosine phosphatase inhibitor, markedly enhanced the high K^+-induced contractile responses, which were attenuated by genistein. The present study also found that sodium orthovanadate could significantly increase the magnitude of contraction in coronary rings in response to KCl, but another interesting finding was that sodium orthovanadate did not affect the relaxation induced by genistein whereas could partly inhibit the dose-dependent vasorelaxation caused by 17-β-estradiol. The results suggest that mechanisms other than tyrosine kinase inhibition are probably responsible for genistein induced-relaxation in porcine coronary arteries. In contrast to genistein, the blockade of 17-β-estradiol induced-relaxation by sodium orthovanadate may be related to its abilities of decreasing NO-mediated vasorelaxation because some papers demonstrated that sodium orthovanadate could decrease eNOS activity through tyrosine phosphorylation-dependent mechanisms in the systemic circulation (Garcia-Cardena et al. 1996), reduce NO-mediated pulmonary vasodilatation and endothelium-dependent relaxation (Huang et al. 2002; Zerrouk et al. 1999). This result was consistent with the observation that the relaxant effect of 17-β-estradiol was partly endothelium-dependent and related to NO in coronary artery rings.

In our experiments, indomethacin did not affect the relaxations induced by genistein and 17-β-estradiol in the coronary artery. The finding indicates that the release of vasodilator prostanoids is not involved in arterial relaxation induced by genistein and 17-β-estradiol.

There are structural similarities between the steroidal nucleus of 17-β-estradiol and the rigid ring structure of the phytoestrogen genistein, and because both of them are lipophilic compounds and their molecular weights are not large, they can easily enter the cytoplasm through cellular membranes to affect expression of some genes. Both the "classical" estrogen receptor ER_α and the novel ER_β 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). Genistein, considered to be a weak estrogen, has been shown to interact with ER_α and ER_β with different affinity and show cardiovascular protective effects. However, in the present experiment, the lack of inhibition of coronary artery relaxations induced by either genistein or 17-β-estradiol after incubation with the estrogen receptor antagonist tamoxifen or the protein synthesis inhibitor cycloheximide suggests that the estrogen receptor probably does not play a role in the acute vasorelaxant effects of genistein and 17-β-estradiol in coronary arteries *in vitro*. Our data support the concepts that the acute vasorelaxant effects caused by genistein and 17-β-estradiol are not mediated by the classical estrogen receptor and are independent of gene-mediated events.

The vasoconstrictive response to KCl is usually used to assess the contractile ability of the vascular smooth muscle (Ren and Zhang 2002). Votage-dependent calcium channels (VDCCs) are activated by depolarization of the plasma membrane when the extracellular K^+ concentration is increased. In our experiment, incubation with genistein and 17-β-estradiol not only shifted the KCl concentration-dependent contraction curves to the right in normal K−H 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 parallel manner. These results are consistent with the effect of genistein and 17-β-estradiol on the big elastic aorta as reported previously (Li et al. 2002; Li et al. 2004) and are supported by the other studies (Nevala et al. 1998; Figtree et al. 2000). In the present experiment, our data suggest that genistein, similar to 17-β-estradiol, may have Ca^{2+}-antagonistic properties and can inhibit extracellular Ca^{2+}-influx through VDCCs. The Ca^{2+} antagonist characteristic of genistein can also be underlined by the previous report that genistein reversibly inhibited L-type calcium current in isolated guinea-pig ventricular myocytes in a concentration-dependent manner (Figtree et al. 2000).

In addition, as we know, 5-HT is released from activated platelets and has an obvious vasocontracting effect which can markedly reduce in the absence of extracellular Ca^{2+} and verapamil (a L-type Ca^{2+} channel blocker), so 5-HT contraction is dependent on voltage-dependent Ca^{2+} channels and transplasmalemmal Ca^{2+} entry (Tasaki et al. 2003). In our experiment we found that both genistein and 17-β-estradiol, just as verapamil (Tasaki et al. 2003), could markedly inhibit the contractile response to 5-HT, and shift its concentration-response curves rightward in a parallel manner. This result also indicates that the inhibition of extracellular Ca^{2+} influx is involved in the vasorelaxing effects of genistein and 17-β-estradiol.

In conclusion, the phytoestrogen genistein can induce significant concentration-dependent relaxation in isolated porcine coronary arteries, which is independent of the classical estrogen receptor and gene expression. The main

mechanism probably involves the inhibition of Ca^{2+}-influx through calcium channels. Also, there is a different mechanism because the relaxing responses of genistein are not related to endothelium and NO, whereas 17-β-estradiol is endothelium-dependent in part. Therefore, genistein has a vasoactivity similar to 17-β-estradiol, and has a potential to replace estradiol in the prevention and treatment of vascular diseases.

4. Experimental

4.1. Tissue preparation

Fresh porcine hearts of either sex were obtained from local abattoir in cold, modified Krebs-Henseleit (K-H) solution of the following composition (in the left anterior descending (LAD) coronary): 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, and cut the proximal LAD (1 cm to 3 cm from the origin) into 4 mm ring segments. Ring samples were then suspended horizontally with two stainless steel hooks in a tissue chamber containing 37 °C oxygenated (95% O_2 and 5% CO_2) K−H solution. Isometric tension generated by vascular smooth muscle was measured using a force transducer (JH-2) and recorded with BL-420 Experimental System of Biological Function (TME, China) with an IBM computer. Resting tension was set to 2 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 with a cotton lot. In all tissues the presence or absence of a functionally intact endothelium was tested by measuring the relaxation response to bradykinin (1 μM) in $PGF_{2\alpha}$(10 μM)-precontracted tissues.

4.2. Experimental protocols

After equilibration, various experiments were done:
(1) Coronary arterial rings with or without endothelium were contracted with 30 mM KCl, when the contractile response had reached a stable plateau (approximately 15–20 min), genistein (1–100 μM), 17-β-estradiol (1–100 μM) or the same volume of solvent were added in progressively increasing cumulative concentrations every 10 min.
(2) In some experiments, rings with endothelium were once again treated with 30 mM KCl and the responses to genistein (1–100 μM) and 17-β-estradiol (1–100 μM) were measured after 20 min preincubation with one of the following specific inhibitors: 100 μM N^{ω}-L-nitro-arginine (L-NNA), 10 μM tamoxifen, 10 μM indomethacin, 10 μM sodium orthovanadate, or 10 μM cycloheximide respectively.
(3) Rings were stabilized at 2 g resting tension for 90 min in K−H solution and the concentration-response curves to 5-HT (0.01–10 μM) were then obtained. After washout of 5-HT, the experiment was repeated in the presence of genistein (30 nM or 1 μM) or 17-β-estradiol (30 nM or 1 μM).
(4) The concentration-response curves to KCl (5–60 mM) were observed in the absence or presence of genistein (1 μM, 30 μM or 100 μM) or 17-β-estradiol (1 μM, 30 μM or 100 μM).
(5) Coronary arterial rings were incubated in calcium-free solution containing 0.01 mM EGTA for 60 min. The calcium concentration-dependent contraction curves were then measured in K^+ depolarization medium (80 mM KCl). Rings were readjusted in normal K−H solution for 10 min and then incubated in calcium-free solution for a further 40 min. After the baseline was stable, the rings were incubated with genistein (30 μM) or 17-β-estradiol (30 μM) for 20 min and the calcium concentration-dependent contraction curves were then obtained again.

4.3. Drugs

Genistein, 17-β-estradiol, N^{ω}-L-nitro-arginine (L-NNA), bradykinin, tamoxifen, indomethacin, sodium orthovanadate, prostaglandin $F_{2\alpha}$($PGF_{2\alpha}$), 5-hydroxytryptamine (5-HT) and cycloheximide (Sigma, Chemical Co, USA); genistein, 17-β-estradiol and tamoxifen were dissolved in dimethylsulfoxide (Me_2SO). The final concentration of Me_2SO in the bath in each case was never more than 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 remaining drug stocks were dissolved in distilled water.

4.4. Data analysis

All results are expressed as mean ± se, n refers to the number of hearts used in the study. Relaxation was expressed as percentage relaxation of contraction induced by KCl (30 mM). In experiments involving concentration-response curves, the results were expressed as percentage of control maximal contractile responses induced by 60 mM KCl, $10^{-5.5}$ M 5-HT or 10 mM $CaCl_2$ respectively. The EC_{50} value of each individual vessel was determined by the Scott Method, and was expressed as negative log molar (pD_2) value. Statistical analysis was performed using Student's *t*-test and analysis of variance (ANOVA). Each group was compared with the solvent control. A probability level of less than 0.05 was considered significant.

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