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Endothelium-dependent relaxation of rat aorta and main pulmonary artery by the phytoestrogens genistein and daidzein

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Abstract

Objective: The dietary phytoestrogens genistein and daidzein have been shown to relax agonist-preconstricted arteries in vitro; the mechanisms of relaxation remain incompletely understood. This study aimed to determine whether the relaxation of phenylephrine (PE)-constricted rat aorta and main pulmonary artery by genistein and daidzein was endothelium-dependent. **Methods:** Effects of endothelial-denudation, and pretreatment with with 100 μM L-N^G-nitroarginine methyl ester (L-NAME) and/or 10 μM indomethacin on relaxation of PE (1 μM)-preconstricted contractures by genistein (1–100 μM) and daidzein (3–100 μM) were assessed by measuring isometric force development by rat arterial rings. The effect of L-NAME on relaxation to 17β-estradiol (10 μM) was also measured in aorta. **Results and conclusions:** Genistein and daidzein caused concentration-dependent relaxation of aorta rings preconstricted with PE (1 μM). The IC_{50} values were 5.7 μM (n=8, 95% confidence limits 4.3–7.7 μM) and 36.7 μM (n=12, 95% confidence limits 25.7–44.1 μM), respectively. Removal of the endothelium and pretreatment with L-NAME (100 μM) significantly inhibited relaxation at 3, 10 and 30 μM genistein and 10 and 30 μM daidzein. The contracture evoked in rat aorta by depolarization with 75 mM K^+ solution was similarly relaxed by genistein in a partially endothelium-dependent manner. 17β-Estradiol (10 μM) caused a 48.7±5.0% (n=11) relaxation of the PE contracture, which was significantly reduced to 25.1±5.3% (n=7) by L-NAME. Relaxations brought about by 17β-estradiol, genistein, and daidzein were not significantly affected by the genomic estrogen receptor antagonist ICI 182,780 (10 μM). Similar endothelium-dependent effects of genistein were observed in the main pulmonary artery. The results show that the relaxation of these rat arteries by concentrations of genistein and daidzein which overlap those present in human plasma after ingestion of soybean-containing meals is largely endothelium dependent

Keywords: Contractile function; Endothelial function; Nitric oxide; Vasoconstriction/dilation

1. Introduction

The isoflavanoids genistein and daidzein are plant-derived estrogen-like compounds (phytoestrogens) which have been shown to relax preconstricted vascular smooth muscle (VSM) [1–3]. Although this effect of genistein may be due to its activity as an inhibitor of non-receptor tyrosine kinases, which are thought to be involved in smooth muscle excitation–contraction coupling [4–7], the analogous effect by daidzein is more difficult to explain, since this substance is considered to be inactive as a tyrosine kinase inhibitor [8]. On the other hand, since there is evidence that estrogen acutely releases nitric oxide (NO) from the endothelium [9], it is possible that relaxation by genistein and daidzein is, at least in part, due to an estrogen-mimetic release of NO. However, most studies of the effects of these phytoestrogens have utilized endothelium-denuded or NOS-inhibitor-treated vessels in order to focus on the role of tyrosine kinases in VSM per se, and

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therefore endothelium-dependent effects may have been missed.

These substances are of particular interest because they are present in high concentration in soybeans, and are therefore dietary constituents, especially in East Asian countries. There is increasing evidence that genistein and related substances exert beneficial effects on the plasma lipoprotein profile (reviewed in [10]), and it has been proposed that genistein might serve as an estrogen-mimetic substance for hormone replacement therapy [11]. King and Bursill [12] recently observed that following ingestion of a soy-based meal, the genistein and daidzein concentrations in plasma reached peak concentrations of ≈4 and 3 μmol, respectively. A recent study also has found that an isoflavanoid-rich diet improved endothelial function in atherosclerotic female primates [11]. In the present study, we have therefore examined the endothelium-dependency of the relaxation of phenylephrine (PE)-preconstricted rat aorta (RA) and rat main pulmonary artery (RPA) by genistein and daidzein.

2. Methods

The investigation conforms with the guide *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Adult male Wistar rats (≈250 g) were killed by cervical dislocation according to UK Home Office guidelines, and the thoracic aorta or left and right branches of main pulmonary artery were isolated. The tissues were placed in cold physiological saline solution (PSS) containing (in mM): NaCl, 118.06; KCl, 4.0; $MgSO_4$, 1; $NaHCO_3$, 24.05; NaH_2PO_4, 0.435; $CaCl_2$, 1.8 and sodium pyruvate, 5.5, gassed with 95% O_2–5% CO_2, and cleaned of fat and connective tissue under a dissecting microscope. Rings of 2–3 mm width were cut and mounted in a 5-ml organ bath containing PSS and continuously gassed with 95% O_2–5% CO_2 at 37°C. In some rings endothelium denudation was achieved by rubbing the luminal side of the vascular rings with a wet cotton swab. The rings thus mounted were subjected to a passive tension of 1 g and equilibrated for 30–45 min with a change of solution every 15 min.

2.1. Experimental protocols

Rings were then stimulated twice for 5 min with a high K^+ solution (similar to PSS except that the KCl concentration was raised to 75 mM, with a corresponding reduction in NaCl) and once with 1 μM PE (10 μM in the RPA), or alternatively with two 5-min exposures to 1 μM PE. To examine the endothelial integrity, acetylcholine or carbachol (1 μM in RA, 2 μM in RPA) was applied at the peak of the final PE contraction. The tissues with intact endothelium responded to the cholinergic agonist with a substantial (typically 30–100%) relaxation; however no relaxation was observed in tissues denuded of endothelium.

Following this run-up procedure, arterial rings were then constricted with either 1 μM PE (aorta) or 10 μM PE (pulmonary artery, which was less sensitive to this agent). Once the contracture had reached a steady level, either genistein or daidzein was added cumulatively in increasing concentrations in order to measure the concentration-dependency of relaxation. Relaxation was measured after it had reached a steady level. Complete reversal of PE-induced contraction was set as 100% relaxation and the percentage relaxation at different concentrations of the phytoestrogens was calculated accordingly.

The role of NO and prostaglandins in relaxation was examined by pretreatment of the endothelium-intact tissues with 100 μM L- N^G-nitroarginine methyl ester (L-NAME) (200 μM L-NAME used in RPA, see below) and/or 10 μM indomethacin. Both indomethacin and L-NAME had their own effects on contraction. In RA, application of L-NAME potentiated the PE contracture, as shown in the upper traces of Figs. 2 and 6. Conversely, indomethacin had little effect on the PE contracture in the RA, and neither drug affected basal tension.

As previously reported [13], L-NAME itself caused a large and sustained contraction of RPA rings. Although the L-NAME induced contraction was smaller at concentrations of this drug <200 μM, lower concentrations also produced a less than maximal inhibition of the acetylcholine-mediated relaxation, and therefore 200 μM L-NAME was used for all studies in RPA. The contraction evoked by 200 μM L-NAME amounted to 84±12% (n=7) of that elicited by high K^+ solution. Subsequent addition of PE (10 μM) in the continuing presence of L-NAME increased the contraction to 157±8% of the high K^+ contraction. In comparison, the contraction to PE alone was 69±13% of the high K^+ response (n=6), and addition of L-NAME in the continuing presence of PE increased the contraction to 186±16% of that evoked by high K^+. Although indomethacin alone did not affect basal tension, when applied to PE-preconstricted RPA it relaxed the PE contraction by 33±5% (n=8).

The endothelium-dependency of the relaxation by genistein of the contracture to 75 mM K^+ solution was also investigated in rat aorta by comparing the effect of genistein in the presence and absence of 100 μM L-NAME and 10 μM. indomethacin. As described above, genistein was added cumulatively in increasing concentrations once the contracture had reached a steady level. In these experiments, however, each concentration of genistein was applied for only 15 min, at which time relaxation had sometimes not reached its maximal level. This procedure, which resulted in a total exposure time to genistein of 75 min, was used because the high K^+ contracture sometimes showed a delayed rundown after several hours. However, time control experiments carried out to assess this effect

under control conditions and in the presence of L-NAME and indomethacin, showed that this effect was negligible during the first 75 min after the contracture had reached a steady level (which typically took about an hour). Tension fell by only 0.5±1.4% and 3.9±2.7% after 30 and 75 min under control conditions (n=6), and by 0.4±0.7 and 0.6±1.8% after 30 and 75 min in the presence of L-NAME and indomethacin (n=6, ns at either time).

2.2. Statistics

With the exception of IC_{50} values, results are depicted as mean±SEM. IC_{50} values obtained from individual concentration–response curves by fitting the points with a sigmoidal function using SIGMAPLOT 5.01 (Jandel, San Rafael, CA, USA), or by linear interpolation, and were then converted to logarithmic values for statistical analysis. IC_{50} values are shown as mean (95% confidence limits). Statistical comparisons were carried out using Student's two tailed t-test for unpaired data, with $P<0.05$ accepted as indicating that the differences between means were significant.

2.3. Drugs

Genistein and daidzein were purchased from Calbiochem–Novabiochem (UK) and 17β-estradiol was from Sigma (Dorset, UK). Stock solutions (100 mM) were prepared in dimethylsulphoxide (DMSO) and the aliquots were stored at −20°C until needed. L-NAME, indomethacin and PE were obtained from Sigma. Stock solutions (10 mM) of acetylcholine, carbachol, L-NAME and PE were prepared in double distilled water and 10 mM stock solution of indomethacin was prepared in DMSO. 17β-Estradiol was dissolved in DMSO at a concentration of 100 mM, and this solution was then diluted in ethanol to produce a 10 mM stock solution of 17β-estradiol. The estrogen receptor antagonist ICI 182,780 was from Tocris Cookson (Bristol, UK) and was prepared as a 10 mM stock solution in DMSO.

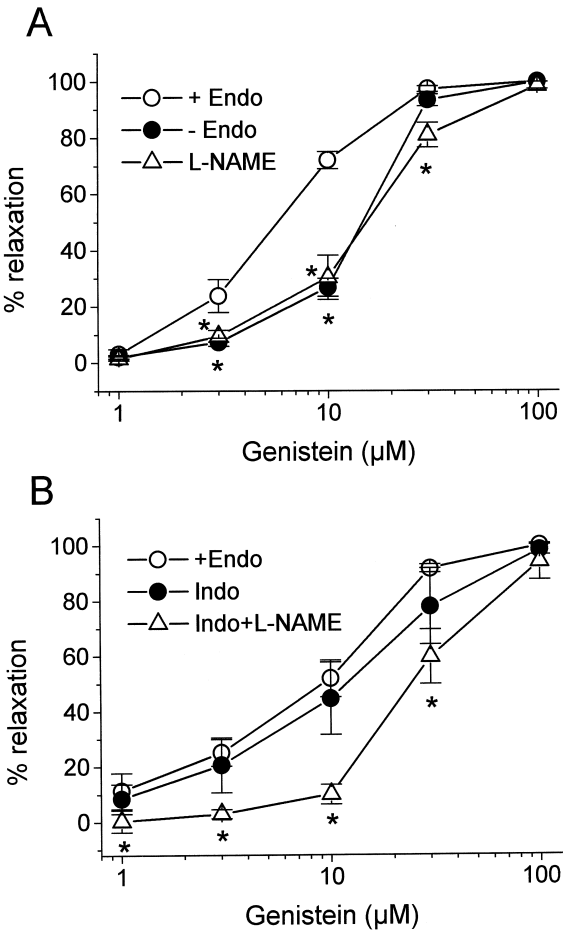

Fig. 1. Genistein-mediated relaxation of the PE contracture in rat aorta is partially endothelium-dependent. (A) Control genistein-induced relaxation of PE (1 μM) constricted rat aorta recorded in endothelium-intact rings (n=8), endothelium-denuded rings (n=7), and endothelium-intact rings pretreated for 30 min with 100 μM L-NAME (n=7). (B) Control genistein-induced relaxation (n=7), compared to genistein-mediated relaxation in rings pretreated with indomethacin (10 μM) (n=5), or indomethacin plus L-NAME (n=7). In this and all subsequent figures asterisks indicate that relaxation under a particular condition was significantly different compared to control. All points (or bars, see Fig. 4) indicate mean±S.E.M. values for percentage relaxation.

3. Results

3.1. Endothelium-dependent relaxation of RA by genistein, daidzein and 17β-estradiol

Fig. 1A shows the concentration-dependency of the relaxation of 1 μM PE preconstricted RA by genistein in endothelium-intact and denuded arteries, and in endothelium-intact arteries in the presence of 100 μM L-NAME. The IC_{50} for relaxation derived by fitting the mean data was 5.7 (4.3–7.7) μM in endothelium-intact rings (n=8), and was increased to 13.4 (11.8–15.2) μM in the endothelium denuded rings (n=7, $P<0.005$) and 14.5 (9.7–21.7) μM in the presence of L-NAME (n=5, $P<0.02$). The relaxation caused by 3 and 10 μM genistein was significantly inhibited both by removal of the endothelium and by L-NAME.

Fig. 1B illustrates the effect of 10 μM indomethacin, and the combination of indomethacin and L-NAME on the relaxation of the RA by genistein. The control genistein IC_{50} was 7.4 (4.6–11.7) μM (n=7) in this set of experiments. Indomethacin did not significantly affect the relaxation to genistein (IC_{50}=10.3 (4.0–26.7) μM, n=5), whereas the combination of indomethacin and L-NAME significantly increased the IC_{50} to 27.3 (17.9–41.9) μM, n=7, $P<0.02$). The relaxation mediated by genistein at 1, 3, 10 and 30 μM was significantly inhibited by the combination of L-NAME and indomethacin. Inhibition of genistein-induced relaxation by L-NAME and in-

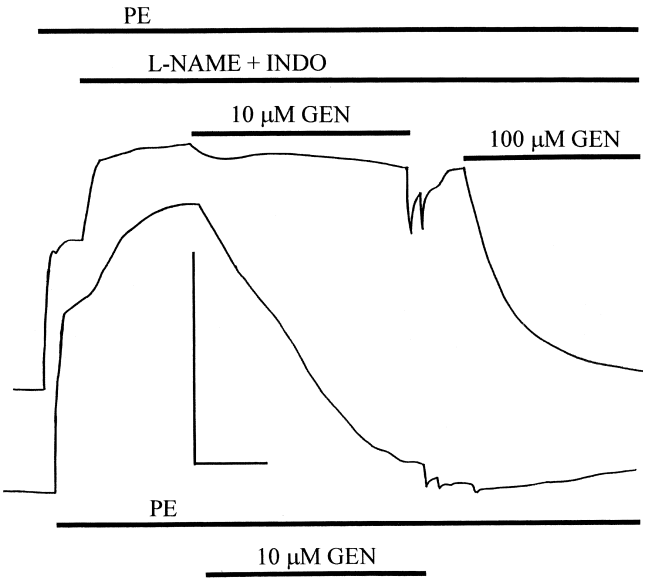

Fig. 2. Suppression of genistein-mediated relaxation of rat aorta by L-NAME and indomethacin. The lower trace (drug additions indicated by bars below this trace) illustrates that 10 μM genistein caused a slowly developing and almost complete relaxation of the PE (1 μM) contraction. The PE contraction failed to recover when genistein was removed by flushing the organ bath several times with PSS, with immediate reapplication of PE. The upper trace (drug additions indicated by bars above the trace) shows that 10 μM genistein caused a much smaller relaxation when applied following pretreatment with L-NAME (100 μM) and indomethacin (10 μM). Following removal of genistein by flushing the organ bath several times with PSS, with immediate reapplication of PE, L-NAME, and indomethacin, application of 100 μM genistein then caused a complete relaxation of the PE contracture. Note that application of L-NAME and indomethacin caused an enhancement of the PE contracture (also visible in the upper trace of Fig. 6). Vertical and horizontal scale bars indicate 1 g tension and 30 min, respectively.

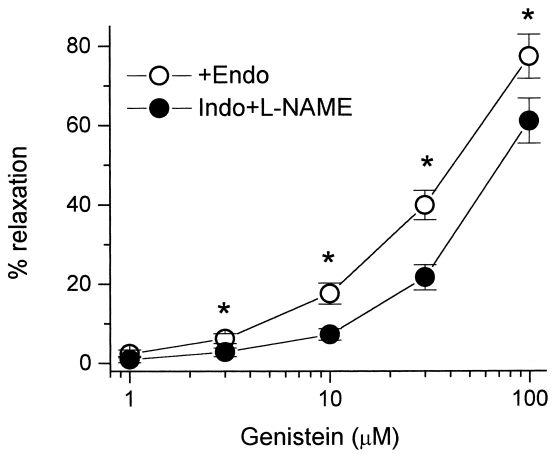

Fig. 3. Genistein-mediated relaxation of the high K^+ contracture in rat aorta is partially endothelium-dependent. (A) Control genistein-induced relaxation of high K^+ (75 mM) constricted rat aorta recorded in endothelium-intact rings (n=9) and endothelium-intact rings pretreated for ≥30 min with 100 μM L-NAME and 10 μM indomethacin (n=10). As described in the Methods, genistein was added cumulatively in ascending concentrations, with each concentration applied for 15 min.

domethacin was especially pronounced at the lower concentrations of genistein; the combination of these drugs reduced relaxation by 97, 88 and 81% at 1, 3 and 10 μM genistein, respectively.

Fig. 2 shows an example of the effect of L-NAME and indomethacin upon the relaxation by genistein of the PE contracture. The lower trace shows that 10 μM genistein caused a slow, almost complete relaxation of the PE contracture when endothelial function was intact. Recovery of the PE contracture following the removal of genistein was very slow and limited. The upper trace shows that the relaxation to 10 μM genistein was almost completely absent in the presence of L-NAME and indomethacin. However, subsequent application of 100 μM genistein in

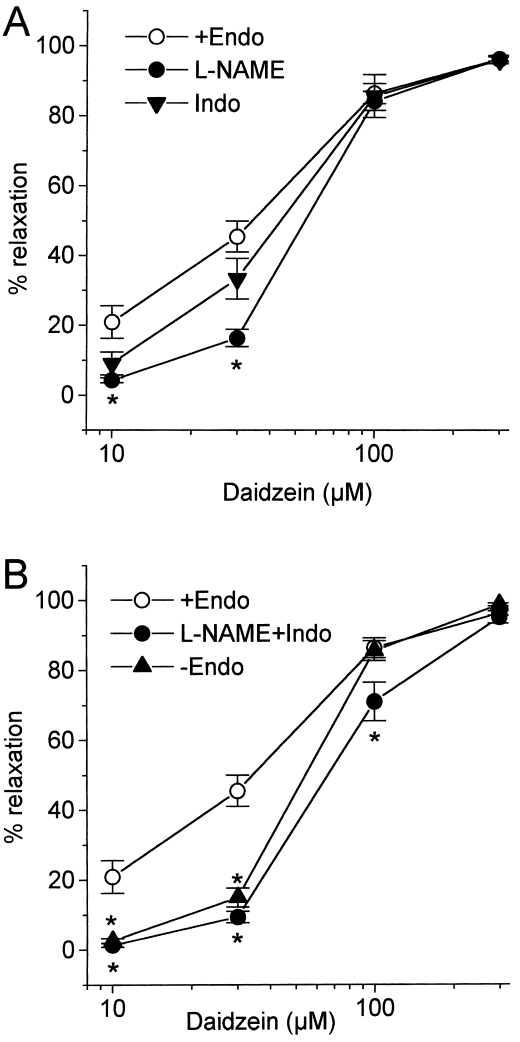

Fig. 4. Daidzein-mediated relaxation of rat aorta is partially endothelium-dependent. (A) Daidzein-mediated relaxation of PE (1 μM) constricted rat aorta recorded in endothelium-intact rings (n=13), endothelium-intact rings pretreated with 100 μM L-NAME (n=7), and endothelium-intact rings pretreated with 10 μM indomethacin (n=8). (B) Comparison of daidzein-induced relaxation under control conditions (n=13, same data as in (A), endothelium-denuded rings (n=6), and after pretreatment with a combination of L-NAME and indomethacin (n=5).

the presence of L-NAME and indomethacin then caused a complete relaxation.

Genistein also caused a concentration-dependent relaxation of the contracture elicited in RA by 75 mM K^+. Fig. 3 shows that the combination of 100 μM L-NAME and 10 μM indomethacin significantly inhibited the relaxation caused by 3, 10, 30 and 100 μM genistein.

The isoflavone daidzein resembles genistein in that it acts as a phytoestrogen [10], but differs from genistein in that it has been shown to lack its tyrosine kinase-inhibitory effect [8]. Daidzein also caused a concentration-dependent relaxation of preconstricted RA (IC_{50}=33.6 (25.7–44.1) μM, n=13) which is illustrated in Fig. 4. Fig. 4A shows that relaxation was significantly inhibited by L-NAME (IC_{50}=55.1 (51.5–59.0), n=7, P<0.02) but not by indomethacin (IC_{50}=41.2 (32.7–51.9) μM, n=8, ns). Endothelial denudation significantly increased the IC_{50} to 54.4 (48.4–61.1) μM. (n=6, P<0.05). The combination of L-NAME and indomethacin had a similar effect (IC_{50}= 67.6 (55.8–82.0) μM, n=5, P<0.05) (Fig. 4B), except that it inhibited relaxation by 10 and 30 μM daidzein to a significantly greater extent than did L-NAME alone. As observed with genistein, effects of blocking endothelial function were more prominent at the lower concentrations of daidzein. For example, the combination of L-NAME and indomethacin reduced relaxation caused by 10 and 30 μM by 94 and 79%, respectively.

If the endothelium-dependency of the relaxation of preconstricted RA by genistein and daidzein is a result of their estrogenicity, it would be predicted that 17β-estradiol should also cause an endothelium-dependent relaxation. Application of 17β-estradiol (10 μM) to PE-preconstricted RA rings caused a progressively developing relaxation which amounted to 47.9±3.8% (n=14) after 30 min. Pretreatment of rings with L-NAME (100 μM), but not the genomic estrogen receptor antagonist ICI 182,780 (10 μM), significantly attenuated this 17β-estradiol-induced relaxation (Fig. 5A). Fig. 5B shows that ICI 182,780 also had no significant effect on the relaxation elicited over 30 min by either genistein (10 μM) or daidzein (30 μM). The lack of effect of ICI 182,780 on relaxations to 17β-estradiol and the two phytoestrogens was confirmed by repeating experiments with two separate lots of the estrogen antagonist.

Fig. 6 illustrates the endothelium-dependency of the relaxation of the PE contracture by 10 μM 17β-estradiol in RA. The lower trace depicts the effect of 17β-estradiol in a ring in which endothelial function was intact, and the upper trace shows that this relaxation was both slowed and attenuated in the presence of L-NAME and indomethacin.

3.2. Endothelium-dependency of the effect of genistein in rat main pulmonary artery

Genistein-mediated relaxation of the PE (10 μM) contraction was also endothelium-dependent in the rat main pulmonary artery. Fig. 7A illustrates that genistein-mediated relaxation was reduced significantly in de-endo-

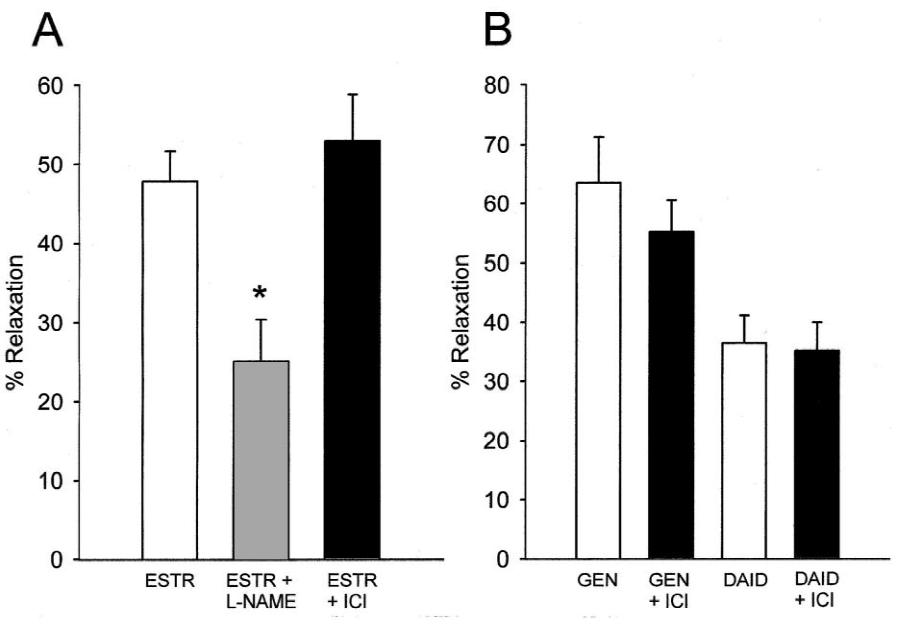

Fig. 5. Relaxation of rat aorta by 17β-estradiol is partially endothelium-dependent, and relaxation by 17β-estradiol and phytoestrogens is not blocked by ICI 780,184. (A) Relaxation of PE-preconstricted RA after 30 min exposure to 17β-estradiol (10 μM) alone (empty bar, n=14), in the presence of L-NAME (100 μM, grey bar, n=7, P<0.01), and in the presence of ICI 780,184 (10 μM, black bar, n=10). Both L-NAME and ICI 780,184 were first applied at least 15 min prior to 17β-estradiol. (B) Relaxation of PE-preconstricted RA after 30 min exposure to 10 μM genistein in the absence (GEN, n=9) or presence of 10 μM ICI 780,184 (GEN+ICI, n=11), and 30 μM daidzein in the absence (DAID, n=7) or presence of 10 μM ICI 780,184 (DAID+ICI, n=7).

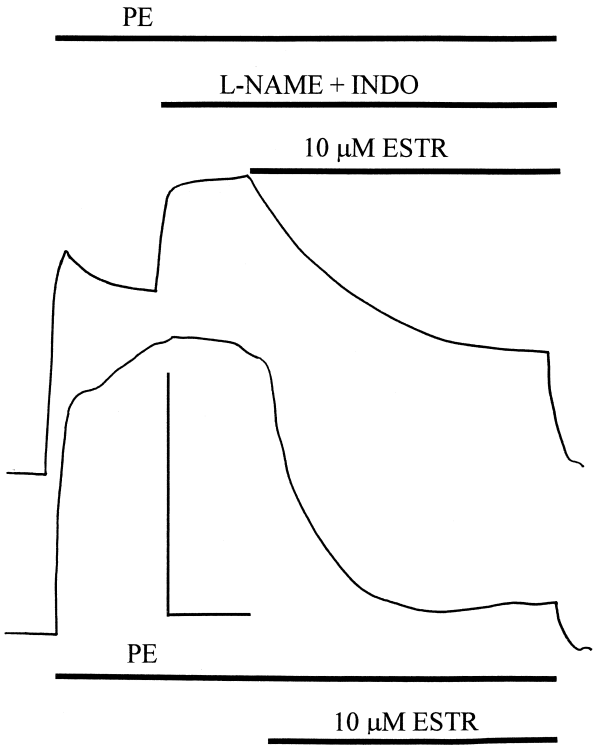

Fig. 6. Inhibition of 17β-estradiol-mediated relaxation of the PE contracture in RA by L-NAME and indomethacin. The lower trace (drug additions indicated by bars below trace) shows the relaxation of the PE contracture by 17β-estradiol (10 μM). The upper trace (drug additions shown above the trace) shows that this relaxation was slower and ultimately smaller in a paired RA ring in which L-NAME (100 μM) and indomethacin (10 μM) were applied before 17β-estradiol. Vertical and horizontal scale bars indicate 1 g tension and 30 min, respectively.

thelialized RPA rings over the entire concentration range examined. IC_{50} values were not calculated as the full range of genistein concentrations was not tested in every ring.

The effect of 200 μM L-NAME and 10 μM indomethacin on the relaxation to genistein was also evaluated in RPA. Fig. 7B shows that L-NAME significantly attenuated relaxation caused by 1, 3, 10 and 30 μM genistein. Conversely, indomethacin (10 μM) did not inhibit relaxation by any concentration of genistein (Fig. 7C).

4. Discussion

These results demonstrate for the first time that the relaxation of VSM by the phytoestrogens genistein and daidzein is partially endothelium-dependent. In particular, relaxation induced by lower concentrations of both genistein (1–10 μM) and daidzein (10 and 30 μM), was largely endothelium-dependent, and in RA was almost abolished by the combination of L-NAME and indomethacin. Relaxation produced by the highest concentrations of genistein and daidzein were less (in RPA) or not at all (in RA) sensitive to endothelial ablation. Higher concentrations of

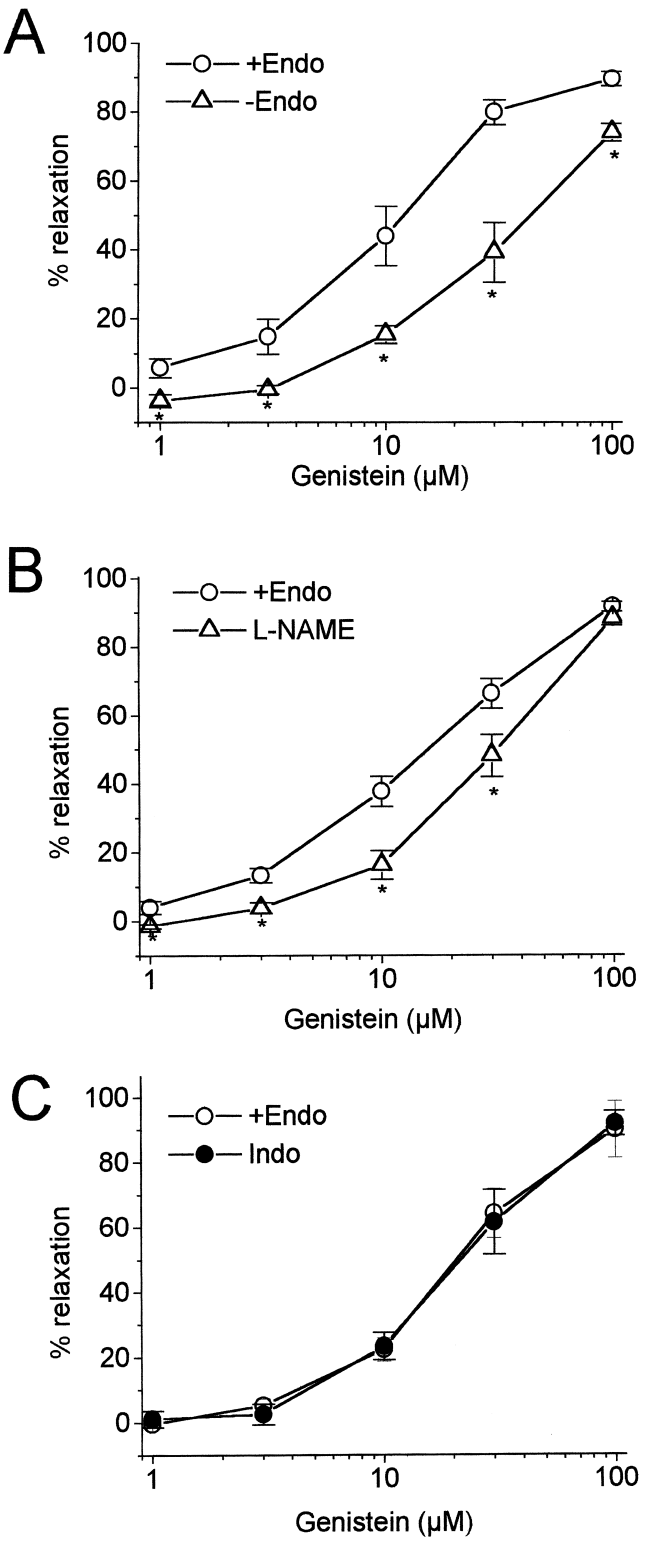

Fig. 7. Genistein-mediated relaxation of rat pulmonary artery is partially endothelium dependent. (A) Control genistein-mediated relaxation of PE (10 μM) constricted RPA recorded in endothelium-intact rings (n=5–7; all concentrations not tested in each ring) and endothelium-denuded rings (n=5–7). (B) Comparison of genistein-induced relaxation under control conditions (n=10–19), and after pretreatment with 200 μM L-NAME (n=6–14). (C) Comparison of genistein-induced relaxation under control conditions (n=4–10), and after pretreatment with 10 μM indomethacin (n=5–10).

these drugs are presumably acting directly on the smooth muscle cells via other pathways, probably including tyrosine kinase inhibition in the case of genistein [4,5,7]. Indomethacin alone caused no significant attenuation of relaxation, implying little role for prostacyclin in this response.

An involvement of the endothelium in the vasorelaxing effects of genistein and daidzein has not previously been reported, probably because most investigators have utilized endothelium-denuded or L-NAME-treated preparations when studying these substances in order to focus on the involvement of tyrosine kinases in mediating vascular relaxation. In a recent study which did address this issue, Nevala et al. [14] demonstrated that neither L-NAME nor indomethacin inhibited relaxation of norepinephrine-preconstricted rat mesenteric artery by genistein and daidzein. However, acetylcholine-induced relaxation in the rat mesenteric artery has previously been shown to be largely insensitive to a combination of the NO synthase inhibitor N^G-nitro-L-arginine and the cyclo-oxygenase inhibitor indomethacin [15], while the relaxation to acetylcholine in both the aorta and pulmonary artery was completely blocked by these agents [15,16]. It would therefore appear that the contribution of endothelium-derived hyperpolarizing factor (EDHF) to endothelium-dependent relaxation is important in the mesenteric artery but not in the aorta. Our results may therefore be reconciled with those of Nevala et al. [14] if these phytoestrogens are enhancing the release of NO, but not of EDHF. The lack of involvement of EDHF in the endothelium-dependent effect of the phytoestrogens is also supported by the observation that L-NAME and indomethacin significantly attenuated the ability of genistein to relax the 75 mM K^+ contracture, which should be insensitive to EDHF (Fig. 3).

As would be predicted if genistein and daidzein were causing endothelium-dependent relaxation by acting at an estrogen receptor, 17β-estradiol also caused a relaxation which was markedly attenuated by L-NAME. A similar endothelium- and NO-dependent relaxation by 17β-estradiol has been reported for isolated coronary and mesenteric resistance arteries of the rat by Otter and Austin [9]. As in this previous study, relaxation began to develop immediately, suggesting that it was not mediated by effects on gene transcription. In agreement with this, we found that the genomic estrogen receptor antagonist ICI 182,780 did not inhibit relaxation by 17β-estradiol, nor did it diminish relaxation by either genistein or daidzein, at concentrations at which the endothelium-dependent component of relaxation was prominent. These results suggest 17β-estradiol, as well as the phytoestrogens, may cause endothelium-dependent relaxation via the putative plasmalemmel estrogen receptor thought to be responsible for the acute affects of this hormone [17].

The relaxation caused by 10 μM 17β-estradiol was roughly equivalent to that caused by the same concentration of genistein with regard to its size, degree of endothelium-dependency, and rate of onset. This suggests that these agents may acutely activate NO synthesis over a similar concentration range. It is noteworthy, however, that this concentration of 17β-estradiol is ≈4 orders of magnitude higher than the concentration normally found in plasma [18], whereas both genistein and daidzein are present in soybeans, and reach plasma concentrations which overlap with those we have found to cause an endothelium-dependent relaxation [12]. There is an increasing recognition of the beneficial cardiovascular effects of soybean isoflavanoids which are usually ascribed to beneficial changes in lipoprotein levels [10]. The present observations suggest, however, that at dietary levels these compounds may also cause NO release. This raises the possibility that a high soy diet might also have effects on the endothelium which might be useful in ameliorating the effects of hypertension, pre-eclampsia, and other cardiovascular diseases.

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