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Chronic treatment of male rats with daidzein and 17β-oestradiol induces the contribution of EDHF to endothelium-dependent relaxation

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1 We investigated the effect of chronic (7 days) treatment of male rats with the isoflavone daidzein (0.2 mg kg^{-1} sc per day) or 17β-oestradiol (0.1 mg kg^{-1} sc per day) on the contribution of nitric oxide (NO), prostaglandins and endothelium-derived hyperpolarising factor (EDHF) to endothelium-dependent relaxation of isolated aortic rings.

2 The sensitivity and maximum relaxation to acetylcholine (ACh) were significantly greater in aortic rings from rats treated with daidzein or 17β-oestradiol, in comparison to vehicle-treated rats. Inhibition of nitric oxide synthase with *N*-nitro-L-arginine (L-NOARG) abolished ACh-induced relaxation in the aortae from vehicle-treated rats, but only attenuated relaxation in aortae from daidzein or 17β-oestradiol-treated rats. The presence of haemoglobin in addition to L-NOARG did not cause any further inhibition of relaxation.

3 The cyclooxygenase inhibitor indomethacin had no effect on endothelium-dependent relaxation in aortae from any treatment group. Charybdotoxin (ChTX), which blocks large-conductance calcium-activated potassium channels (BK_{Ca}) and intermediate-conductance calcium-activated potassium channels (IK_{Ca}), plus apamin, which blocks small-conductance calcium-activated potassium channels (SK_{Ca}), but not iberiotoxin, which only blocks BK_{Ca}, attenuated endothelium-dependent relaxation of aortae from daidzein or 17β-oestradiol-treated rats. Blockade of K_{Ca} channels had no effect on the responses to ACh in aortae from vehicle-treated rats. In aortae from daidzein- or 17β-oestradiol-treated rats, endothelium-dependent relaxation was also attenuated by inhibition of cytochrome *P*450 (CY*P*450) epoxygenase with 6-(2-propargylloxyphenyl)hexanoic acid (PPOH) or inhibition of K_{IR} channels and Na^+/K^+-ATPase with barium and oubain, respectively.

4 This study demonstrates that endothelium-dependent relaxation of male rat aorta is normally entirely mediated by NO, whereas treatment with daidzein or 17β-oestradiol stimulates a contribution from a non-NO, nonprostaglandin factor acting through the opening of SK_{Ca} and IK_{Ca} channels, and involving activation of Na/K-ATPase, K_{IR} and CY*P*450 epoxygenase. This pattern of sensitivity to the tested inhibitors is consistent with the contribution of EDHF to relaxation. Thus, EDHF contributes to the enhanced endothelium-dependent relaxation that is observed after chronic treatment with the phytoestrogen daidzein or with 17β-oestradiol.
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Abbreviations: ACh, acetylcholine; ANOVA, analysis of variance; BK_{Ca}, large-conductance calcium-activated potassium channels; ChTX, charybdotoxin; CY*P*450, cytochrome *P*450; DMSO, dimethyl sulphoxide; EDHF, endothelium-derived hyperpolarising factor; EETs, epoxyeicosatrienoic acids; eNOS, endothelial nitric oxide synthase; IBTX, iberiotoxin; IK_{Ca}, intermediate-conductance calcium-activated potassium channels; K_{IR}, inwardly rectifying potassium channels; KPSS, high potassium physiological saline solution; L-NOARG, *N*-nitro-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; PPOH, 6-(2-propargyloxyphenyl)hexanoic acid; SK_{Ca}, small-conductance calcium-activated potassium channels

Introduction

There is increasing evidence that oestrogen improves endothelial function, in particular by increasing the expression and/or activity of endothelial nitric oxide synthase (eNOS) (Koh, 2002; Mendelsohn, 2002). Thus, oestrogen may increase the bioactivity of NO, leading to the beneficial outcomes of vasodilatation and inhibition of adhesion of platelets and leukocytes to the endothelium. The cyclooxygenase product prostacyclin is also a potent vasodilator and antiaggregatory agent, and oestrogen is reported to increase the expression of cyclooxygenase and prostacyclin synthase (Mendelsohn & Karas, 1999). Furthermore, the activity of endothelium-derived hyperpolarising factor (EDHF), a non-NO, non-cyclooxygenase product, may also be influenced by oestrogen. For example, oestrogen deficiency decreases EDHF-mediated relaxation of rat mesenteric arteries (Liu *et al*., 2001), whereas pregnancy enhances the contribution of EDHF to rat mesenteric artery dilatation (Gerber *et al*., 1998). Importantly, prostacyclin and EDHF act to maintain flow-induced dilation

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of rat gracilis arterioles when NO release is impaired by chronic treatment with a NOS inhibitor (Huang *et al*., 2001). In that study, oestrogen was found to increase the contribution of EDHF to endothelium-dependent dilatation.

Although these beneficial actions of oestrogen indicate a potential for protection against vascular disease, recent clinical trials using oestrogen alone, or in combination with progesterone, show no benefit on the incidence of coronary artery disease, and provoke an increased incidence of stroke (Beral *et al*., 2002; Grady *et al*., 2002; Writing Group WHI, 2002). Moreover, further significant impediments to the therapeutic use of oestrogen are the development of breast and endometrial cancers in women, and unacceptable effects on reproductive function in men. Development of a vascular selective alternative to oestrogen therapy is highly desirable, and this has led to interest in the plant-derived oestrogen mimetics such as the isoflavones. Legumes, in particular soybeans, are an excellent source of isoflavones, and diets rich in soy products reduce several risk factors for cardiovascular disease in primates and humans (Anthony *et al*., 1996; Nestel, 2003). Isoflavones, such as genistein and daidzein, mimic the ability of oestradiol to improve endothelium-dependent relaxation after ovariectomy in rats (Squadrito *et al*., 2000), but they do not affect reproductive tissues in either males or females (Anthony *et al*., 1996; Mitchell *et al*., 2001). Thus, there is evidence that isoflavones may be a suitable alternative to oestrogens to prevent vascular disease; however, the mechanism by which they may enhance endothelial function has not been elucidated. The endothelium synthesises and releases several factors that cause relaxation of the underlying smooth muscle, that is, nitric oxide, prostacyclin and, the as yet unidentified, EDHF. Although isoflavones have been reported to enhance endothelium-dependent relaxation (Anderson *et al*., 1999; Squadrito *et al*., 2000; 2002), there are no reports regarding the selective effects upon individual mediators of that relaxation. This raises the question as to whether isoflavones are able to increase the contribution of prostacyclin and/or EDHF to endothelium-dependent relaxation. The aim of this project was to further investigate the mechanism by which chronic treatment with the isoflavone daidzein or 17β-oestradiol enhances endothelium-dependent relaxation by determining whether they affect the contribution of NO, cyclooxygenase products and EDHF to the endothelium-dependent relaxation of male rat aorta.

Methods

Preparation of rat aorta

Male Sprague–Dawley rats (250–300 g) were housed three to a cage, with free access to tap water and food pellets. The rats were killed by exposure to 80% CO_2/20% O_2 for 5 min. The descending thoracic aorta was dissected and placed in Krebs-bicarbonate solution of the following composition (mM): NaCl 118, $Na_2H_2CO_3$ 25.0, glucose 11.0, $CaCl_2$ 1.6, KCl 4.7, KH_2PO_4 1.2 and $MgSO_4$ 1.18. The superficial connective tissue and fat surrounding the aorta were removed and the aorta was cut in 3–4 mm long ring segments. The rings were mounted between stainless-steel hooks, with one hook linked to an isometric force transducer (model # FT03, Grass Medical Instruments), which was connected to a chart recorder (model # R-02, Rikadenki Kogyo Co.). The other hook was connected to a glass rod in a 10 ml organ bath chamber containing Krebs-bicarbonate solution maintained at 37°C with a pH of 7.4, and continuously aerated with 95% O_2 and 5% CO_2. Aortic rings were allowed to equilibrate for 90 min at a resting tension of 0.5 g, with the bath medium changed every 20 min. All preparations were maximally contracted with isotonic, high potassium salt solution (KPSS, 123 mM). Integrity of he endothelium was confirmed when acetylcholine (ACh, 10 μM) caused greater than 70% relaxation of the phenylephrine (PE, 100 nM) precontracted rings.

Treatment with daidzein or 17β*-oestradiol*

Rats were treated with daidzein (0.2 mg kg^{-1} per day), 17β-oestradiol (0.1 mg kg^{-1} per day) or vehicle (10% DMSO, 0.1 ml) by subcutaneous injection for 7 days. This dose of 17β-oestradiol has previously been shown to improve endothelial function in ovariectomised female rats (Anderson *et al*., 1999), and daidzein was given on an equimolar basis. After 7 days of treatment, the rats were killed and aortic rings were dissected and mounted in organ baths, as described above. The effect of the treatment on relaxant responses was examined by cumulative concentration–response curves to ACh (100 nM–10 μM), to tissues precontracted submaximally with the thromboxane mimetic U-46619 (10–50 nM). All concentration–response curves were undertaken in the presence of nifedipine (10 nM) to inhibit spontaneous contractile activity. In addition, responses to ACh were examined after treatment with *N*-nitro-L-arginine (L-NOARG, 100 μM), haemoglobin (20 μM), indomethacin (10 μM), iberiotoxin (IBTX, 100 nM), 6-(2-propargyloxyphenyl)hexanoic acid (PPOH, 100 nM), a combination of charybdotoxin (ChTX, 100 nM) and apamin (10 nM) or barium (Ba^{2+}, 30 μM) plus oubain (0.1 mM).

Dugs and chemicals used

Acetylcholine perchlorate (BDH Chemicals Poole, U.K.), apamin (Bachem, Bubendorf, Germany), barium chloride (Sigma, St Louis, MO, U.S.A.), ChTX (Bachem), daidzein (Indofine, Belle Mead, NJ, U.S.A.), haemoglobin (Sigma), indomethacin (Sigma), IBTX (Bachem), N^G-nitro-L-arginine (Sigma), 17β-oestradiol (Sigma), oubain (Sigma) and phenylephrine hydrochloride (Sigma) were dissolved in distilled water. Daidzein (Indofine) and 17β-oestradiol (Sigma) were dissolved in 10% DMSO: 90% milli Q water and PPOH (Sigma) was dissolved in ethanol. 9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$ (U46619, Sigma) was dissolved in ethanol (100%) as a stock solution (1 mM), and further dilutions were in distilled water.

Data presentation and statistical analyses

All results are expressed as the mean $\pm$ s.e.m., with *n* indicating the number of experiments. For the effect of extended treatment on relaxant contractile function, concentration–response curves from rat isolated thoracic aorta were computer fitted to a sigmoidal curve using nonlinear regression (Prism version 3.0, GraphPad software, U.S.A.) to calculate the agonist sensitivity (pEC_{50}). Maximum relaxation (Rmax) to ACh was measured as a percentage of precontractions to PE, and contractile responses were measured as a percentage of maximal contraction with KPSS. The calculated pEC_{50} and

Rmax were compared using one-way analysis of variance (ANOVA), with *post hoc* multiple comparisons using Bonferroni's test (Prism version 3.0, GraphPad software, U.S.A.).

Results

Effect of daidzein or 17β-oestradiol treatment on relaxation to ACh and SNP

The relaxant responses to ACh in aortic rings from rats treated with daidzein 17β-oestradiol or vehicle are shown in Figure 1. The sensitivity to ACh in aortic rings from rats treated with daidzein (pEC_{50} 7.51$\pm$0.08%) or 17β-oestradiol (pEC_{50} 7.49$\pm$0.07%) was significantly greater than in aortae from vehicle-treated rats (pEC_{50} 7.16$\pm$0.06%, $P<0.05$, Bonferroni test). In addition to an increase in the potency of ACh, daidzein or 17β-oestradiol treatment significantly enhanced maximum relaxation (Rmax, vehicle 89$\pm$2%, daidzein 99$\pm$1%, 17β-oestradiol 100$\pm$1%, $P<0.05$ compared to vehicle, Bonferroni test). SNP-induced relaxation was similar in aortic rings from all treatment groups, with no change in sensitivity (pEC_{50} vehicle 8.59$\pm$0.11%, daidzein 8.73$\pm$0.08%, 17β-oestradiol 8.78$\pm$0.19%) or maximum relaxation (vehicle 100$\pm$1%, daidzein 99$\pm$1%, E2 99$\pm$1%). Contractile responses to 123 mM KPSS were similar in aortic rings from vehicle (2.25$\pm$0.06 g, $n=36$), daidzein- (2.22$\pm$0.06 g, $n=34$) and 17β-oestradiol- (2.26$\pm$0.05 g, $n=32$) treated rats.

Effect of daidzein or 17β-oestradiol on relaxation to ACh in the presence of L-NOARG

In the presence of L-NOARG, the relaxation to ACh in aortic rings from vehicle-treated rats was totally abolished (Table 1), indicating that the relaxation is entirely NO dependent. In contrast, in arteries from rats treated with daidzein- or 17β-oestradiol, ACh-induced relaxation, although significantly reduced (Table 1), was still present after treatment with L-NOARG alone or in combination with haemoglobin. This indicates that there is a non-NO factor that contributes to ACh-induced relaxation in aorta from rats treated with daidzein or 17β-oestradiol.

Effect of indomethacin on ACh-induced relaxation after daidzein or17β-oestradiol treatment

Indomethacin did not affect relaxation to ACh in aortic rings from vehicle, daidzein- or 17β-oestradiol-treated rats (Table 1). Furthermore, indomethacin did not affect the residual relaxation that was apparent after L-NOARG treatment in aortic rings from daidzein- or 17β-oestradiol-treated rats (Table 1).

Effect of IBTX or ChTX plus apamin on ACh-induced relaxation after daidzein or 17β-oestradiol treatment

IBTX, a blocker of BK_{Ca}, did not affect relaxant responses to ACh in aortic rings from vehicle, daidzein- or 17β-oestradiol-treated rats (Table 2). Furthermore, IBTX plus L-NOARG did not change ACh relaxation responses when compared to L-NOARG alone (Table 2). In contrast, the combination of ChTX plus apamin, blockers of BK_{Ca}/IK_{Ca} and SK_{Ca}, respectively, significantly inhibited ACh-induced relaxation in aortic rings from daidzein- or 17β-oestradiol-treated rats, but not from vehicle-treated rats (Figure 2, Table 2). The combination of ChTX, apamin plus L-NOARG abolished the ACh-induced relaxation of aortae from daidzein- and 17β-oestradiol-treated rats.

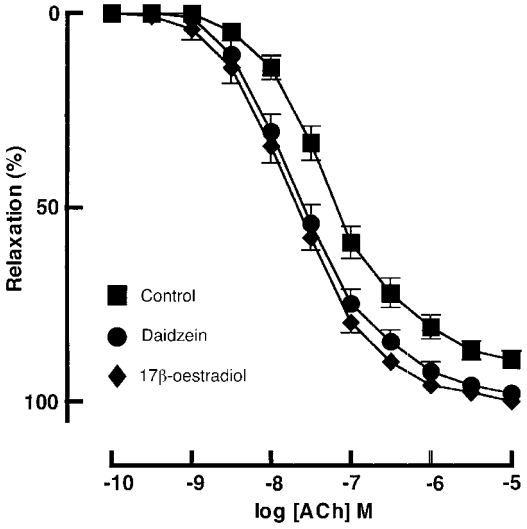

Figure 1 Concentration–response curves to ACh in endothelium intact aortic rings removed from rats treated with daidzein (0.2 mg kg^{-1} per day s.c., $n=15$); 17β-oestradiol (0.1 mg kg^{-1} per day s.c., $n=17$) or vehicle ($n=21$) for 7 days. Aortic rings were precontracted with U46619 to an equal level in all groups (50$\pm$1% of KPSS). Results are shown as mean$\pm$s.e.m.

Table 1 Effect of *N*-nitro-L-arginine, haemoglobin and indomethacin on acetylcholine-induced relaxation of aortae from vehicle, daidzein- and 17β-oestradiol-treated rats

	Vehicle			*Daidzein*			*17β-oestradiol*		
Ach	n	*pEC_{50}*	*Rmax (%)*	n	*pEC_{50}*	*Rmax (%)*	n	*pEC_{50}*	*Rmax (%)*
Control	6	7.10$\pm$0.08	89$\pm$4	6	7.32$\pm$0.1*	99$\pm$1*	6	7.34$\pm$0.04*	96$\pm$2*
L-NOARG	6	ND	0$^{\#}$	6	5.82$\pm$0.4$^{\#}$	28$\pm$6$^{\#}$*	6	5.93$\pm$0.19$^{\#}$	33$\pm$5$^{\#}$*
L-NOARG + Hb	6	ND	0$^{\#}$	6	6.33$\pm$0.3$^{\#}$	29$\pm$4$^{\#}$*	6	6.16$\pm$0.26$^{\#}$	28$\pm$3$^{\#}$*
Indomethacin	6	7.12$\pm$0.10	87$\pm$4	6	7.26$\pm$0.09	95$\pm$2*	6	7.25$\pm$0.12	94$\pm$3*
Indomethacin + L-NOARG	6	ND	0$^{\#}$	6	6.55$\pm$0.09$^{\#}$	21$\pm$10$^{\#}$*	6	6.64$\pm$0.31$^{\#}$	29$\pm$4$^{\#}$*

A comparison of the sensitivity (pEC_{50}) and maximum relaxation to ACh in the absence (control) and presence of *N*-nitro-L-arginine (L-NOARG, 100 μM) alone, or with haemoglobin (Hb, 20 μM) in endothelium intact thoracic aortae from vehicle, daidzein- (0.2 mg kg^{-1} per day s.c.) and 17β-oestradiol- (0.1 mg kg^{-1} per day s.c.) treated rats. n = the number of experiments. *Significantly different from control ACh response in aortic rings from vehicle-treated rats (Bonferroni test, $P<0.05$). $^{\#}$Significantly different from the corresponding control ACh response within the same treatment group (Bonferroni test, $P<0.05$). ND = could not be calculated.

Table 2 Effect of *N*-nitro-L-arginine and potassium channel blockers on acetylcholine-induced relaxation of aortae from vehicle, daidzein- and 17β-oestradiol-treated rats

	Vehicle			*Daidzein*			*17β-oestradiol*		
ACh	n	pEC_{50}	*Rmax (%)*	n	pEC_{50}	*Rmax (%)*	n	pEC_{50}	*Rmax (%)*
Control	10	7.13±0.09	86±3	10	7.34±0.07*	98±0.7*	10	7.35±0.06*	97±2*
L-NOARG	10	ND	0#	10	5.92±0.40#	28±6*#	10	5.94±0.14#	30±3*#
IBTX	6	7.16±0.07	86±4	6	7.25±0.10	92±3	6	7.34±0.07	92±4
IBTX + L-NOARG	6	ND	0	6	6.48±0.10	24±1*#	6	5.84±0.30	25±5*#
ChTX + apamin	4	7.15±0.18	83±3	4	6.99±0.14#	66±8*#	4	6.70±0.3#	54±11*#
ChTX, apamin + L-NOARG	4	ND	0	4	ND	0#	4	ND	0#

A comparison of the sensitivity (pEC_{50}) and maximum relaxation (Rmax) to ACh in the absence (control) and presence of *N*-nitro-L-arginine (L-NOARG, 100 μM), iberiotoxin (IBTX, 100 nM), IBTX plus L-NOARG, charybdotoxin (ChTX, 100 nM) and apamin (10 nM) or ChTX, apamin plus L-NOARG in isolated thoracic aortae from vehicle, daidzein- (0.2 mg kg^{-1} per day s.c.) and 17β-oestradiol- (0.1 mg kg^{-1} per day s.c.) treated rats. n = the number of experiments. *Significantly different from control ACh response in aortic rings from vehicle-treated rats (Bonferroni test, $P<0.05$). #Significantly different from corresponding control ACh response within the same treatment group (Bonferroni test, $P<0.05$). ND = could not be calculated.

Effect of PPOH on ACh-induced relaxation

PPOH, the selective inhibitor of the epoxygenation reactions catalysed by specific cytochrome *P*450 (CY*P*450) isozymes, significantly inhibited ACh-induced relaxation in aortic rings from daidzein- and 17β-oestradiol-treated rats (Figure 3), but not from vehicle-treated rats (data not shown). The maximum relaxation to ACh was significantly reduced in aortic rings taken from rats treated with daidzein (Rmax control 97±2, PPOH 80±8%, $P<0.05$, Bonferroni test) or 17β-oestradiol (Rmax control 99±1, PPOH 75±7%, $P<0.05$, Bonferroni test). The presence of PPOH plus L-NOARG further attenuated ACh-induced relaxation (Rmax daidzein treated 15±3%, 17β-oestradiol treated 0±0%, $P<0.05$, Bonferroni test).

Effect of barium plus oubain on ACh-induced relaxation

A combination of Ba^{2+} and oubain, blockers of K_{IR} and Na^+/K^+-ATPase, respectively, did not alter the relaxation responses to ACh in aortic rings from vehicle-treated rats (data not shown), but inhibited endothelium-dependent relaxation in the aortae from rats treated with daidzein or 17β-oestradiol (Figure 3). In the presence of Ba^{2+} plus oubain, there was a significant reduction in the maximum relaxation to ACh in aortic rings from rats treated with daidzein (Rmax control 95±1, Ba^{2+} and oubain 85±3%, $P<0.05$, Bonferroni test) or 17β-oestradiol (Rmax control 99±1, Ba^{2+} and oubain 81±3%, $P<0.05$, Bonferroni test). Furthermore, the combination of Ba^{2+} and oubain plus L-NOARG caused additional significant inhibition of ACh-induced relaxation (Rmax daidzein treated 0±0%, 17β-oestradiol treated 11±5%, $P<0.05$, Bonferroni test).

Discussion

This study demonstrates that treatment of male rats with the isoflavone daidzein or 17β-oestradiol for 7 days selectively enhances endothelium-dependent relaxation of the isolated aorta. Furthermore, daidzein and 17β-oestradiol treatment stimulates the contribution of a non-NO, noncyclooxygenase product to endothelium-dependent relaxation that is not apparent in the aortae from vehicle-treated rats. After treatment with daidzein or 17β-oestradiol, endothelium-dependent relaxation was attenuated by combined inhibition of SK_{Ca} and IK_{Ca} channels, but not by a selective inhibitor of BK_{Ca} channels. This pattern of sensitivity to selective inhibitors of specific K_{Ca} channels is consistent with the contribution of an as yet unidentified EDHF (Busse *et al*., 2002; Figure 4). This is further supported by our findings that endothelium-dependent relaxation of the rat aorta was sensitive to inhibition by a combination of Ba^{2+} and oubain, blockers of K_{IR} and Na^+/K^+-ATPase, respectively, and by PPOH, a selective inhibitor of CY*P*450 epoxygenase. Importantly, once again, this was only observed in arteries from rats treated with daidzein or 17β-oestradiol. Our studies indicate that EDHF, in addition to NO, contributes to endothelium-dependent relaxation in male rats after treatment with the isoflavone or oestrogen.

Three factors have been demonstrated to mediate endothelium-dependent relaxation, that is, NO, the arachidonic acid metabolite, PGI_2 and the as yet unidentified EDHF. In normal rats, endothelium-dependent relaxation of the male rat aorta was entirely mediated by NO, as the response to ACh was abolished by the NOS inhibitor L-NOARG. In contrast, after rats were treated with daidzein or 17β-oestradiol for 7 days, there was a component of the endothelium-dependent relaxation that was resistant to the NOS inhibitor. As oestrogen and phytoestrogens are reported to increase NO activity (Squadrito *et al*., 2000; Mendelsohn, 2002), we investigated whether the remaining relaxation reflected greater NOS activity which was consequently able to overcome the effect of the NOS inhibitor. The addition of haemoglobin to L-NOARG did not cause any further attenuation of the ACh-induced relaxation, indicating that daidzein and 17β-oestradiol treatment stimulate the contribution of a non-NO factor to endothelium-dependent relaxation. In addition to NO, the endothelium is known to release the arachidonic acid metabolite prostacyclin, another potent vasodilator. The contribution of prostaglandins to endothelium-dependent relaxation may be modulated by the level of NOS activity as well as hormonal status. For example, in the gracilis muscle arterioles of male mice, there is an increased contribution of a cyclooxygenase product to endothelium-dependent dilatation in eNOS knockout mice (Sun *et al*., 1999). In addition, oestrogen treatment has been reported to enhance the release of prostacyclin from the aorta of ovariectomised rats (Bolego *et al*., 1997). However, in this

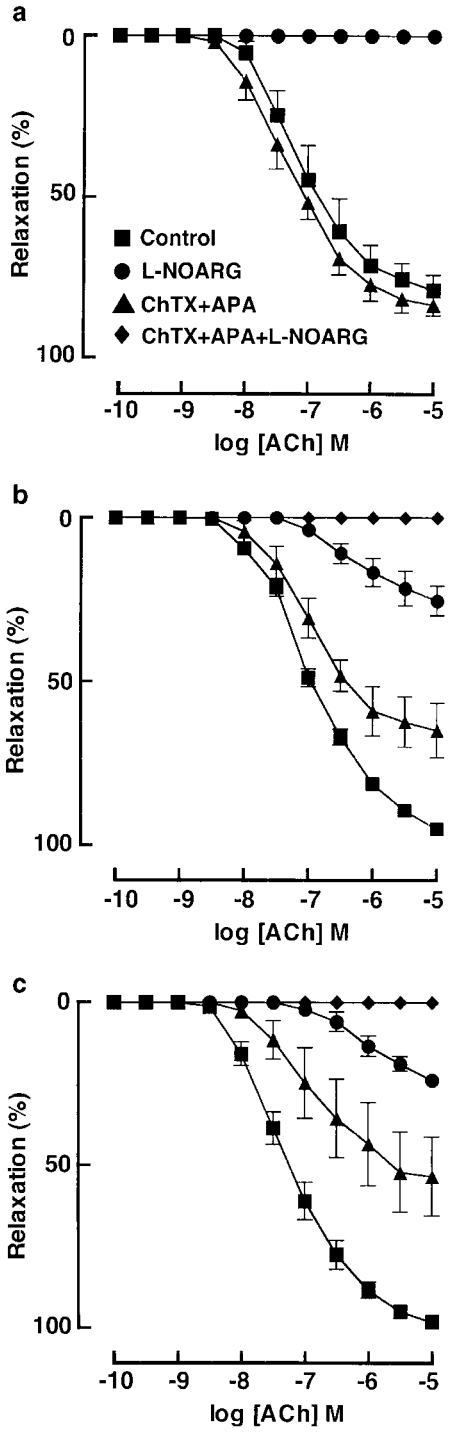

Figure 2 Cumulative concentration–response curves to ACh in the absence (control) or presence of L-NOARG, ChTX plus APA or ChTX, apamin plus L-NOARG in endothelium intact aortic rings from rats treated for 7 days with vehicle (a), daidzein (0.2 mg kg^{-1} per day s.c., $n=4$ (b)) or 17β-oestradiol (0.1 mg kg^{-1} per day s.c., $n=4$ (c)). Aortic rings were precontracted with U46619 to an equal level in all groups (vehicle 52$\pm$2%, daidzein 51$\pm$2%, 17β-oestradiol 50$\pm$1% of KPSS). Results are shown as mean$\pm$s.e.m. The pEC_{50} and R_{max} values determined from the data presented in these graphs are given in Table 2.

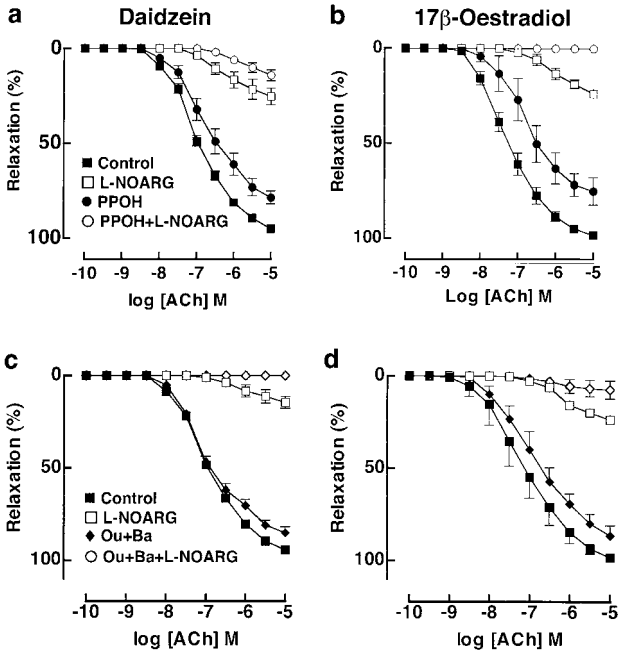

Figure 3 Cumulative concentration–response curves to ACh in aortic rings from rats treated for 7 days with daidzein (0.2 mg kg^{-1} per day s.c., $n=4$ (a, c)) or 17β-oestradiol (0.1 mg kg^{-1} per day s.c., $n=4$ (b, d)). Endothelium-dependent relaxation in response to ACh was determined in the absence and presence of L-NOARG, PPOH or L-NOARG plus PPOH (A and B) or L-NOARG, oubain (Ou) plus barium (Ba) or oubain, barium plus L-NOARG (c, d). Aortic rings were precontracted with U46619 to an equal level in all groups ((a) 52$\pm$1%, (b) 52$\pm$2%, (c) 52$\pm$1%, (d) 51$\pm$1% of KPSS).

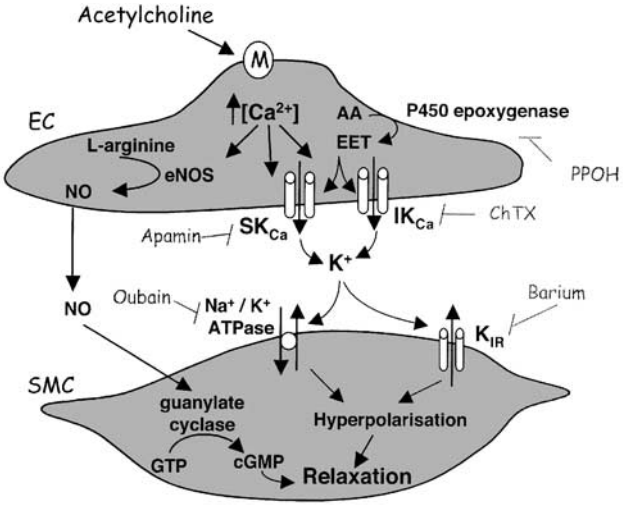

Figure 4 Proposed mechanism of endothelium-dependent relaxation in aortae removed from male rats treated with daidzein or 17β-oestradiol for 1 week. In the aortae from vehicle-treated rats, ACh-induced relaxation was entirely mediated by NO. In contrast, after treatment with daidzein or 17β-oestradiol, the relaxant response to ACh was attenuated by apamin plus ChTX, indicating the involvement of the opening of SK_{Ca} and IK_{Ca} channels on endothelial cells. Sensitivity of the relaxation to oubain and barium further indicates a role for the Na^+/K^+-ATPase and K_{IR} channels, each of which is found on smooth muscle. It has been suggested that EETs, products of *P*450 epoxygenase, facilitate the opening of endothelial K_{Ca} channels by enhancing Ca^{2+} influx or by increasing the sensitivity of those channels to Ca^{2+}. (See the text for more detail and references.)

study, prostaglandins did not mediate ACh-induced relaxation, as the cyclooxygenase inhibitor indomethacin had no effect on endothelium-dependent relaxation in either normal rats or after treatment with daidzein or 17β-oestradiol. A contribution of prostaglandins to endothelium-dependent relaxation is sometimes evident only after inhibition of NOS (Wu *et al*., 2001). That was not observed in this study, as indomethacin had no further effect on the relaxation remaining in the presence of NOS inhibition in vessels from the daidzein- or 17β-oestradiol-treated rats.

EDHF is the third potential contributor to endothelium-dependent relaxation that was investigated. EDHF has not been definitively identified and the mechanism by which it causes relaxation remains a subject of debate. Busse *et al*. (2002) have recently reviewed the proposed mechanisms of EDHF-mediated relaxation, concluding that several mechanisms may be operating simultaneously, depending on the tissue. Clearly, however, there is strong evidence for the involvement of calcium-activated potassium (K_{Ca}) channels and the activation of CY*P*450, with the resultant generation of epoxyeicosatrienoic acids (EETs) (Figure 4). We therefore investigated whether inhibitors of these mechanisms influenced endothelium-dependent relaxation of the aorta from rats treated with daidzein or 17β-oestradiol. IBTX, an inhibitor of BK_{Ca}, did not affect responses to ACh under any conditions. In contrast, the combination of ChTX, which blocks BK_{Ca} and IK_{Ca}, plus apamin, which blocks SK_{Ca}, significantly attenuated endothelium-dependent relaxation in aortae from daidzein- and 17β-oestradiol-treated rats, but not from vehicle-treated rats. Both IK_{Ca} and SK_{Ca} channels are expressed by endothelial cells but not smooth muscle cells, including the rat aorta (Marchenko & Sage, 1996), whereas BK_{Ca} are found on the vascular smooth muscle but not on the endothelium (Neylon *et al*., 1999; Quignard *et al*., 2000). Busse *et al*. (2002) have suggested that the hyperpolarisation of endothelial cells arising from the activation of IK_{Ca} and SK_{Ca} channels may be regulated by the activation of CY*P*450 and the subsequent synthesis of EETs (Figure 4). Our finding that PPOH, an inhibitor of CY*P*450 epoxygenase, attenuates ACh-induced relaxation of aortae from daidzein- and oestrogen-treated rats supports a role for EETs in endothelium-dependent relaxation. PPOH is a relatively selective inhibitor of CY*P*450 epoxygenase (Wang *et al*., 1998) that has previously been used to identify the contribution of EETs to endothelium-dependent vasodilatation (Huang *et al*., 2001). EETs have been suggested to facilitate the opening of endothelial K_{Ca} channels by enhancing the entry of Ca^{2+} in response to Ca^{2+} store depletion (Hoebel *et al*., 1997) and/or increasing the sensitivity of endothelial K^+ channels to Ca^{2+} (Baron *et al*., 1997).

The endothelium-dependent relaxation of aortae from daidzein- and 17β-oestradiol-treated rats was also sensitive to attenuation by the combination of Ba^{2+} plus oubain, to inhibit K_{IR} and Na^+/K^+-ATPase, respectively. This suggests that the efflux of K^+ through the endothelial IK_{Ca} and SK_{Ca} channels could then elicit hyperpolarisation of the vascular smooth muscle by activation of K_{IR} and Na^+/K^+-ATPase (Figure 4). Although we have not measured the membrane potential in these experiments, all of the results are consistent with the contribution of EDHF to endothelium-dependent relaxation, but only when the rats had been treated with the isoflavone or oestrogen.

The relative contribution of NO, prostaglandins and EDHF to endothelium-dependent dilatation is influenced by a number of factors including vessel size (Garland *et al*., 1995), gender and hormonal status (Golding & Kepler, 2001; Sato *et al*., 2002). In this study, using a large artery from untreated male rats, endothelium-dependent relaxation was entirely mediated by NO as responses to ACh were abolished by inhibition of NOS and ChTX plus apamin had no effect on relaxation. This is consistent with previous studies in which EDHF has been reported to have little or no contribution to endothelium-dependent relaxation of the tail artery (Pak *et al*., 2002) or mesenteric arteries (Mcculloch & Randall, 1998) from male rats. In the same preparations from female rats, EDHF made a significant contribution to endothelium-dependent relaxation. The results of this study indicate that treatment with daidzein or 17β-oestradiol can induce the contribution of EDHF to endothelium-dependent relaxation in male rats contributing to enhanced ACh-induced relaxation.

This study demonstrates that chronic treatment with daidzein (0.2 mg kg^{-1} per day s.c.) caused a similar level of enhancement of the potency and maximum relaxation to ACh as 17β-oestradiol (0.1 mg kg^{-1} per day s.c.). The dose used has previously been shown to cause plasma levels of 17β-oestradiol in OVX rats similar to those seen in intact, nonpregnant rats (Anderson *et al*., 1999). Daidzein was administered on an approximately equimolar basis. In contrast to this similar efficacy in relation to improving endothelial function, the binding affinity of 17β-oestradiol to ERα or ERβ is 200–1000 times higher than for daidzein (Kuiper *et al*., 1998). While there may be many factors influencing the level of activity of each compound, for example, differences in pharmacokinetics, this result is suggestive of a lack of involvement of ERα or ERβ in the vascular effects of daidzein or 17β-oestradiol. This is consistent with reports that improvement of endothelial function in response to acute application of these compounds is unaffected by the non-selective oestrogen receptor antagonist ICI 182,780 (Karamsetty *et al*., 2001).

In conclusion, chronic treatment of male rats with daidzein or 17β-oestradiol enhances endothelium-dependent relaxation in response to ACh. Whereas responses to ACh were normally entirely mediated by NO, daidzein and 17β-oestradiol induced a component of the endothelium-dependent relaxation that was resistant to NOS inhibition. The non-NO component of the relaxation was not mediated by a cyclooxygenase product, but was sensitive to inhibition of IK_{Ca} and SK_{Ca} channels. In addition, we provide evidence that an EET is involved in the response, and the activation of K_{IR} channels and Na^+/K^+-ATPase contributes to the relaxation. All of these observations are consistent with the contribution of EDHF (Busse *et al*., 2002) to endothelium-dependent relaxation. Daidzein was found to be equally effective to 17β-oestradiol in enhancing endothelial function. As isoflavones have been demonstrated to lack effect on the reproductive function of males or females (Anthony *et al*., 1996; Mitchell *et al*., 2001), daidzein, or related isoflavones, may be an alternative treatment to oestrogens in the prevention of vascular disease that can be safely used in males as well as females.

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