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Pharmacological profile of phytoestrogens in cerebral vessels: in vitro study with rabbit basilar artery

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Abstract

As a previous step to consider their use in the pharmacology for stroke, we investigated the effects of four phytoestrogens (i.e. genistein, daidzein, zearalanone and biochanin A) on cerebral vessels. Cerebral vascular responses were analyzed by conventional recording of isometric tension in rabbit basilar artery segments kept in organ bath under standard conditions. The four phytoestrogens elicited concentration-dependent relaxant responses of different potency in basilar artery segments previously contracted with either 5×10^{-2} M KCl or 10^{-4} M UTP. Neither endothelium removal, 10^{-4} M N^{ω}-nitro-L-arginine methyl ester (L-NAME, nitric oxide (NO) synthase inhibitor), 10^{-5} M1 *H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ, selective inhibitor of NO-sensitive guanylyl cyclase), 10^{-5} M 4*H*-8-bromo-1,2,4-oxadiazolo(3,4-*d*)benz(*b*)(1,4)oxazin-1-one (NS2028, specific soluble guanylyl cyclase inhibitor), nor 10^{-5} M indomethacin (prostaglandin biosynthesis inhibitor) modified the phytoestrogen-elicited vasorelaxant responses. On the other hand, Ca^{2+}-elicited contractile responses were effectively inhibited in the presence of phytoestrogens. Phytoestrogens act as cerebrovascular relaxants by a mechanism which involves Ca^{2+} entry blockade in the vascular smooth muscle rather than stimulation of vasorelaxant endothelium-related mechanisms such as NO/cGMP or prostaglandins.

Keywords: Phytoestrogen; Cerebral artery; Ca^{2+} channel; Nitric oxide (NO); Estrogen; Stroke

1. Introduction

Consideration of estrogen as a potential therapy for stroke is a matter of controversy. Evidence in favour of its use are the following: (1) epidemiological studies have shown that stroke is rare among premenopausal women and its overall incidence much lower than in age-matched men, and also that this condition is gradually lost after menopause; and (2) a large amount of in vivo animal studies suggest that treatment with estrogen improves outcome after cerebral ischemia (for reviews, see Hurn and Macrae, 2000; Roof and Hall, 2000). Evidence against its use comes from epidemiological studies with estrogen replacement therapy. The use of estrogen replacement therapy in the primary prevention of cardiovascular diseases in postmenopausal women is based on the early conviction that endogenous estrogen affords protection against cardiovascular disease in premenopausal women. However, two randomized trials have failed to find benefits from estrogen replacement therapy in women with pre-existing disease, and show estrogen replacement therapy-related undesirable effects such as an increase in the risk of breast cancer and an increase in vaginal bleeding (Mosca et al., 2001; Mikkola and Clarkson, 2002; Russo et al., 2002). As the Stroke Council of the American Heart Association states, until more data are available, the impact of estrogen replacement therapy on stroke risk should be considered almost null (Goldstein et al., 2001). This has taken to search for alternatives to estrogen replacement therapy.

Phytoestrogens are naturally occurring plant-derived nonsteroidal estrogens which are present in the human

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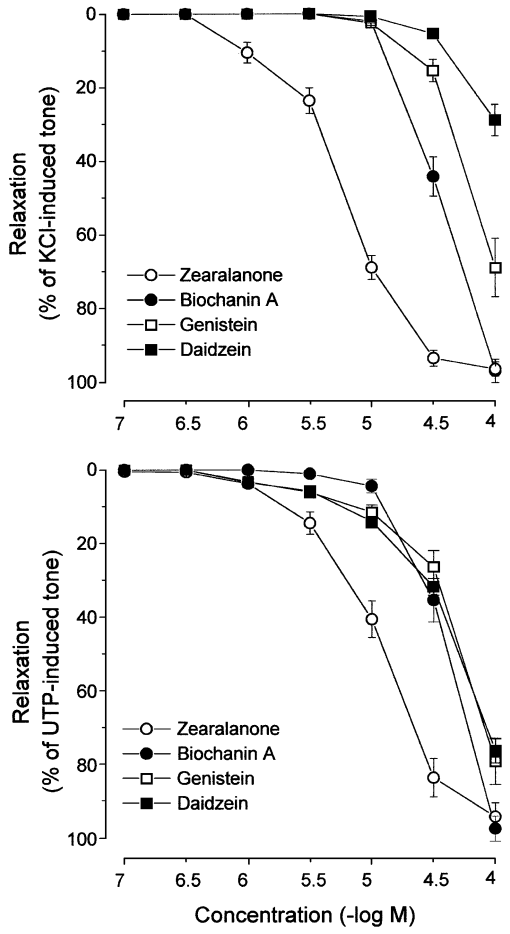

Fig. 1. Relaxant responses of rabbit basilar artery exposed to the four phytoestrogens investigated and previously contracted with 5×10^{-2} M KCl (upper panel) or 10^{-4} M UTP (lower panel). Values are mean ± S.E.M.

diet. Their chemical structure is similar to that of estrogen, what enables them to bind the estrogen receptor thus acting as estrogen agonists or antagonists (for reviews, see Setchell, 1998; Belcher and Zsarnovszky, 2001). Interest in phytoestrogens comes from the observation that people from East Asian countries showed a lower incidence of cardiovascular diseases than people from Western countries. Since Asians consume 20–50 times more soy-derived food per capita than Americans do, and soybeans are a concentrated source of phytoestrogens (particularly isoflavones), it has been postulated that phytoestrogens could be responsible for the health-promoting effect of soy consumption (for reviews, see Glazier and Bowman, 2001; Bhathena and Velasquez, 2002; Stark and Madar, 2002; Wang, 2002).

The therapeutic possibilities of phytoestrogens have received much attention in last years. The pharmacological effects of phytoestrogens are assumed to result mainly from their ability to inhibit the catalytic activities of many enzymes, as well as their ability to scavenge free radicals (for review, see De Groot and Rauen, 1998). Phytoestrogens have been recognized to possess antiinflammatory, antioxidant, antithrombotic, antineoplasic, antiallergic and hepatoprotective activities (for review, see Middleton et al., 2000). With regard to the central nervous system, dietary flavonoid-type phytoestrogens are now emerging as potential neuroprotectants against several brain pathologies including ischemia–reperfusion, although the exact mechanisms of neuroprotection are unknown (for review, see Youdim et al., 2002).

We try to challenge the hypothesis that phytoestrogens could be useful tools in the prevention and/or treatment of stroke by its ability to alter vascular reactivity and cerebral blood flow in a similar way as estrogen does. We have recently reported that 17-β-estradiol relaxes cerebral arteries due to inhibition of extracellular Ca^{2+} influx to vascular smooth muscle (Salom et al., 2001). As a first step to assess that hypothesis, in the present work, we have studied the reactivity of rabbit-isolated basilar artery during acute exposure to four phytoestrogens: genistein, daidzein, biochanin A and zearalanone. Specifically, we studied: (1) the effect of the four phytoestrogens on vascular tension at resting or active tone; (2) the possible involvement of endothelium-related mechanisms such as the nitric oxide (NO)/cGMP pathway and prostanoid synthesis in the responses induced by the phytoestrogens; and (3) the ability of the four phytoestrogens to inhibit Ca^{2+}-elicited contractile responses.

Table 1
Pharmacological parameters of the concentration–response relaxant responses from the four phytoestrogens on rabbit-isolated basilar artery

	KCl (5×10^{-2} M)			UTP (10^{-4} M)		
	EC_{50} ($\times 10^{-5}$ M)	E_{max} (%)	n	EC_{50} ($\times 10^{-5}$ M)	E_{max} (%)	n
Genistein	4.67 (4.26–5.13)	69 ± 8[a]	13	4.07 (3.55–4.68)	79 ± 6	16
Daidzein	5.01 (4.78–5.24)	29 ± 4	14	3.71 (3.55–3.89)	76 ± 3	14
Zearalanone	0.57 (0.48–0.70)[b]	96 ± 2[c]	14	1.14 (0.91–1.44)[b]	94 ± 4[d]	13
Biochanin A	3.38 (2.81–4.07)[e]	97 ± 3[c]	14	3.89 (3.38–4.46)	97 ± 3[f]	16

EC_{50} values are expressed as mean with its 95% confidence limits (in parenthesis) from "n" arterial segments. E_{max} values are expressed as percentage of previous contraction to 5×10^{-2} M KCl or 10^{-4} M UTP in the same arterial segment, and represented as mean ± S.E.M. from "n" arterial segments.

[a] Significantly higher than daidzein, $P<0.001$. Student–Newman–Keuls test.

[b] Significantly lower than genistein, daidzein and biochanin A, $P<0.001$. Student–Newman–Keuls test.

[c] Significantly higher than genistein and daidzein, $P<0.001$. Student–Newman–Keuls test.

[d] Significantly higher than genistein and daidzein, $P<0.05$. Student–Newman–Keuls test.

[e] Significantly lower than genistein, $P<0.01$, and daidzein, $P<0.001$. Student–Newman–Keuls test.

[f] Significantly higher than genistein, $P<0.05$, and daidzein, $P<0.01$. Student–Newman–Keuls test.

2. Materials and methods

Experiments were conducted in compliance with the Spanish legislation on ‘‘Protection of Animals used for Experimental and other Scientific Purposes’’, and in accordance with the Directives of the European Community on this subject. Experimental protocols were revised and approved by the Ethical Committee of the Hospital ‘‘La Fe’’.

2.1. Tissue preparation

Eighty five New Zealand White male rabbits (Technology Transferring Centre, Polytechnic University of Valencia, Spain), weighing 2.5–3 kg, were killed by injection of 25 mg/kg sodium thiopental (Tiobarbital, B Braun Medical, Jaén, Spain) and 1.5 ml of 10^{-2} M KCl solution through the ear vein. The whole brain, including the brainstem, was removed and the basilar artery was dissected free. Four 3-mm-long segments of basilar artery were obtained. Some segments were mechanically devoid of endothelium by gentle rubbing with a stainless steel rod introduced through the arterial lumen. For isometric tension recording, the segments were mounted in an organ bath by using tungsten wires (89 μm in diameter). Two pins were introduced through the arterial lumen. One pin was fixed to a stationary support, while the other pin was connected to a strain gauge (Universal Transducing Cell UC3, Gould Statham, Oxnard, CA, USA). Isometric tension was conveniently amplified (Hewlett-Packard 8805D, San Diego, CA, USA) and recorded (Omniscribe D5237-5, Houston Instrument, Gistel, Belgium). Each organ bath contained 5 ml of Ringer-Locke solution at 37 °C and bubbled with a 95% O_2 and 5% CO_2 mixture to give a pH of 7.3–7.4. Previously determined optimal resting tension of 0.5 g was applied to the arterial segments, and then they were allowed to equilibrate for 30–60 min before starting the experiments.

2.2. Experimental procedure

The contractile capacity of every arterial segment was assessed by exposure to 5×10^{-2} M KCl Ringer-Locke solution. Those segments contracting less than 0.5 g were discarded. Cumulative concentration–response curves to genistein, daidzein, zearalanone and biochanin A were obtained in arteries at resting or active tone elicited with 5×10^{-2} M KCl or with 10^{-4} M UTP. To assess the role of endothelium in the effects of phytoestrogens, responses were elicited in some rubbed, KCl-precontracted basilar artery segments. The functional state of the endothelioum was verified by challenge of KCl-precontrated arteries with acetylcholine (10^{-6}, 10^{-5} and 10^{-4} M). In order to assess the possible involvement of NO/cGMP- or prostaglandin-related mechanisms in the phytoestrogen-elicited response, arterial segments were preincubated with either 10^{-4} M N^{ω}-nitro-L-

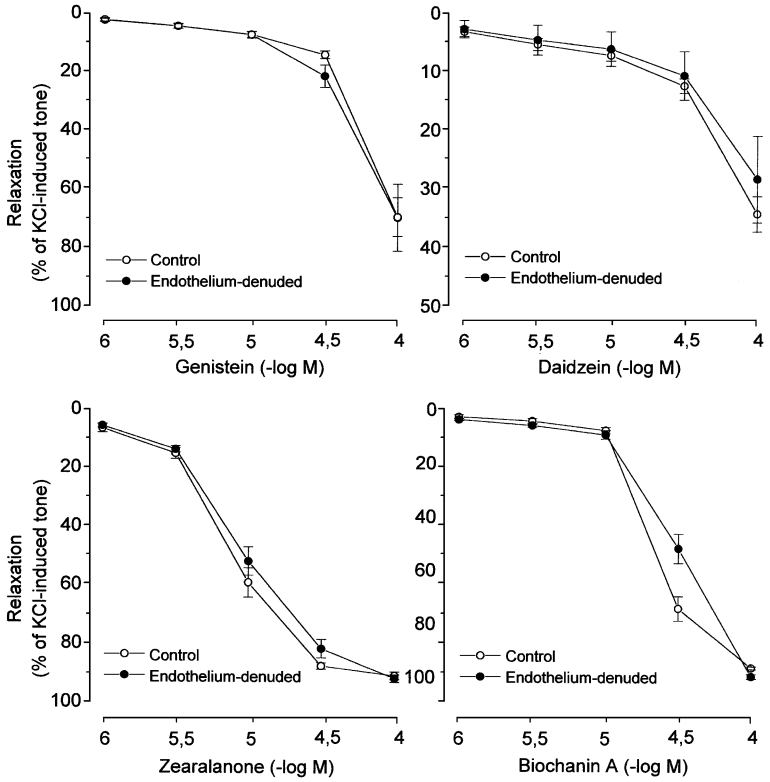

Fig. 2. Influence of endothelium on the relaxant responses of KCl (5×10^{-2} M)-precontracted rabbit basilar artery to genistein (upper left panel), daidzein (upper right panel), zearalanone (lower left panel) and biochanin A (lower right panel). Values are mean ± S.E.M.

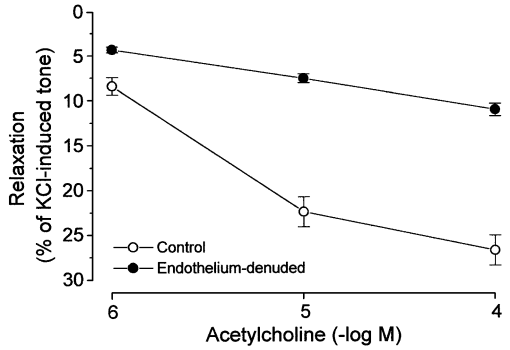

Fig. 3. Influence of endothelium on the relaxant responses of KCl (5×10^{-2} M)-precontracted rabbit basilar artery to acetylcholine. Values are mean ± S.E.M.

arginine methyl ester (L-NAME, NO synthase inhibitor), 10^{-5} M 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ, selective inhibitor of NO-sensitive guanylyl cyclase), 10^{-5} M 4*H*-8-bromo-1,2,4-oxadiazolo(3,4-*d*)benz (*b*)(1,4)oxazin-1-one (NS2028, specific soluble guanylyl cyclase inhibitor), or 10^{-5} M indomethacin (prostaglandin biosynthesis inhibitor). On the other hand, the ability of phytoestrogens to modulate Ca^{2+} entry was assessed by obtaining concentration–response curves to $CaCl_2$ (10^{-5}–10^{-2} M) in the absence or in the presence of two concentrations of the phytoestrogens (10^{-5} and 10^{-4} M). For this purpose, basilar arteries were washed three times (10-min interval) with Ca^{2+}-free medium containing 10^{-3} M ethylene glycol-bis[β-aminoethyl ether]-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA). Then, arteries were stimulated with Ca^{2+}-free, 5×10^{-2} M KCl medium (without or with phytoestrogens) and cumulative concentrations of $CaCl_2$ were added.

2.3. Data analysis

The relaxant responses elicited by genistein, daidzein, zearalanone and biochanin A were expressed as percentage of the active tone achieved with KCl or UTP. The contractile responses to $CaCl_2$ were expressed as percentage of previous response to KCl before Ca^{2+} washing. Maximum effect (E_{max}) and half-maximal effective drug concentration (EC_{50}) were calculated for each concentration–response curve. The pEC_{50} was calculated as the negative logarithm to base 10 of the EC_{50} for statistical analysis. Except for zearalanone, these pharmacological parameters are 'apparent' for phytoestrogens because no real E_{max} was detected at the highest concentrations used. One-way analysis of variance (ANOVA) followed by Student–Newman–Keuls multiple comparison test was used to compare the effects of the four phytoestrogens without or with treatments, as well as to compare the effects of $CaCl_2$ in phytoestrogen-treated arteries, at the different concentrations used, with the effects of $CaCl_2$ in their respective control groups. The Student's unpaired *t*-

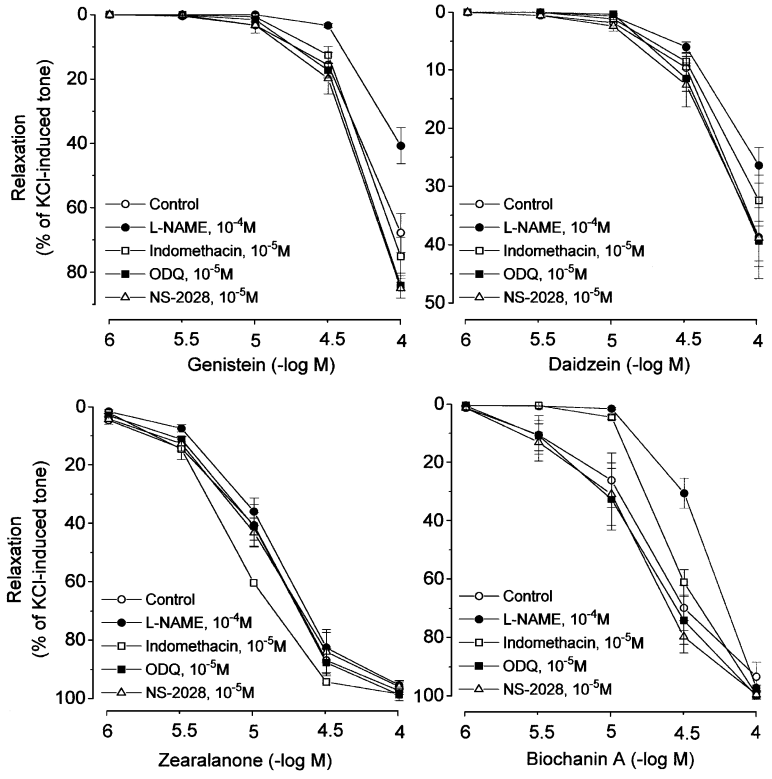

Fig. 4. Influence of NO/cGMP- or prostaglandin-related mechanisms on the relaxant responses of KCl (5×10^{-2} M)-precontracted rabbit basilar artery to genistein (upper left panel), daidzein (upper right panel), zearalanone (lower left panel) and biochanin A (lower right panel). Values are mean ± S.E.M.

Table 2
NO/cGMP and prostanoid-related mechanisms in the relaxant responses elicited by phytoestrogens in rabbit-isolated basilar artery

	Genistein			Daidzein			Zearalanone			Biochanin A		
	EC_{50} ($\times 10^{-5}$ M)	E_{max} (%)	n	EC_{50} ($\times 10^{-5}$ M)	E_{max} (%)	n	EC_{50} ($\times 10^{-5}$ M)	E_{max} (%)	n	EC_{50} ($\times 10^{-5}$ M)	E_{max} (%)	n
Control	4.67 (4.46–4.89)	68 ± 6	12	4.67 (4.57–4.78)	39 ± 5	12	1.09 (0.91–1.31)	97 ± 1	12	1.54 (1.17–2.04)	93 ± 5	12
L-NAME, 10^{-4} M	5.24 (5.12–5.37)	41 ± 6[a]	9	4.46 (4.16–4.78)	26 ± 3	9	1.34 (1.17–1.54)	95 ± 1	9	4.16[b,c] (3.89–4.46)	97 ± 1	9
ODQ, 10^{-5} M	4.78 (4.67–4.89)	84 ± 4	9	4.67 (4.36–5.01)	39 ± 3	9	1.20 (1.09–1.31)	98 ± 2	9	1.47 (1.12–1.94)	99 ± 1	9
NS2028, 10^{-5} M	4.57 (4.26–4.89)	85 ± 3	9	4.26 (4.07–4.46)	39 ± 7	9	1.20 (1.00–1.44)	96 ± 2	9	1.28 (1.00–1.65)	99 ± 1	9
Indomethacin, 10^{-5} M	5.01 (4.89–5.13)	75 ± 6	9	4.57 (4.46–4.67)	32 ± 4	9	0.79 (0.72–0.87)	98 ± 2	9	2.39 (2.08–2.75)	99 ± 1	9

EC_{50} values are expressed as mean with its 95% confidence limits (in parenthesis) from "n" arterial segments. E_{max} values are expressed as percentage of previous contraction to 5×10^{-2} M KCl in the same arterial segment, and represented as mean ± S.E.M. from "n" arterial segments.

[a] Significantly lower than Control, ODQ, NS2028 and Indomethacin groups, $P < 0.001$. Student–Newman–Keuls test.

[b] Significantly higher than Control and NS2028 groups, $P < 0.01$. Student–Newman–Keuls test.

[c] Significantly higher than ODQ group, $P < 0.05$. Student–Newman–Keuls test.

test was used to compare the effects of acetylcholine and the four phytoestrogens in intact and rubbed arteries. A P value of 0.05 or less was considered significant.

2.4. Drugs and solutions

Genistein, daidzein, zearalanone, biochanin A, L-NAME, ODQ, NS2028, EGTA and indomethacin were from RBI-Sigma-Aldrich Química (Alcobendas, Spain). The phytoestrogens were dissolved (10^{-2} M) in dimethyl sulfoxide (DMSO). Total DMSO added to the organ bath for cumulative concentration–responses or preincubations was, at the most, 0.34% v/v and did not affect arterial tone. The Ringer-Locke solution had the following composition ($\times 10^{-3}$ M): NaCl 120, KCl 5.4, $CaCl_2$ 2.2, $MgCl_2$ 1.0, $NaHCO_3$ 25 and glucose 5.6. In Ca^{2+}-free medium, $CaCl_2$ was omitted from the composition, and, when indicated, EGTA, was added. In KCl-depolarizing

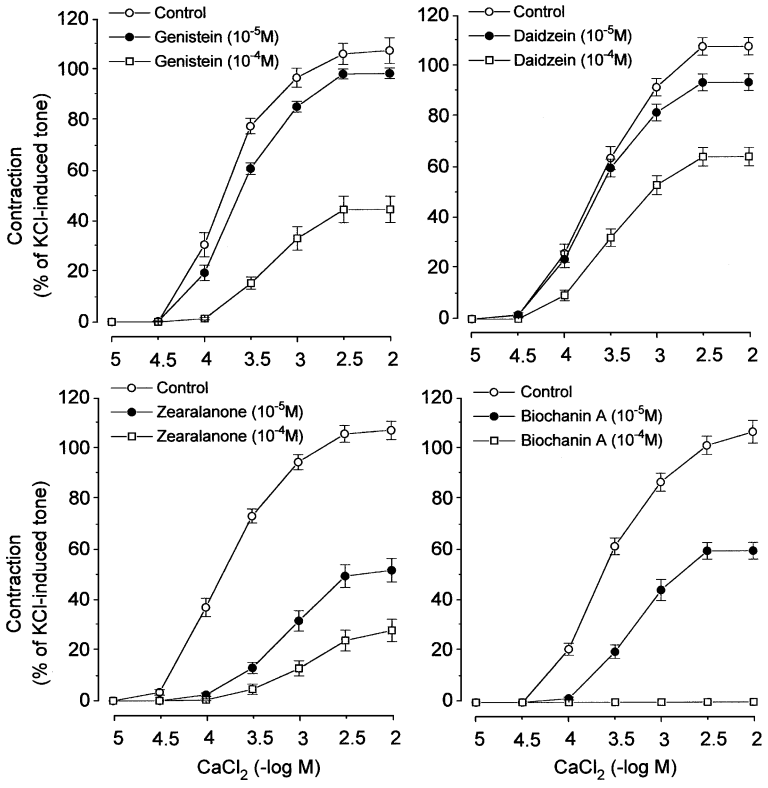

Fig. 5. Ca^{2+}-elicited responses in rabbit basilar artery preincubated with genistein (upper left panel), daidzein (upper right panel), zearalanone (lower left panel) and biochanin A (lower right panel). Values are mean ± S.E.M.

solution, NaCl was replaced by an equimolar amount of KCl.

3. Results

Cumulative addition of increasing concentrations of phytoestrogens (10^{-7}–10^{-4} M) did not elicit measurable changes in arterial segments at resting tone. By contrast, the four phytoestrogens relaxed in a concentration-dependent manner the arterial segments previously contracted with either 5×10^{-2} M KCl or 10^{-4} M UTP (Fig. 1). The EC_{50} and E_{max} values from the concentration-dependent relaxant responses to the four phytoestrogens are shown in Table 1. The statistical analysis of the data showed different relationships among the phytoestrogens depending on the pharmacological parameter (EC_{50} or E_{max}) and the precontractile agent (KCl or UTP) considered. When the arterial segments were precontracted with KCl, the following order of potency (EC_{50} comparison) was found: zearalanone>biochanin A>genistein = daidzein. The order of efficacy (E_{max} comparison) was: biochanin A = zearalanone>genistein>daidzein. When the arterial segments were precontracted with UTP, the order of potency found was: zearalanone>daidzein = biochanin A = genistein. The order of efficacy was: biochanin A = zearalanone>genistein = daidzein.

Mechanical rubbing of the endothelium did not significantly modify the relaxant responses elicited by phytoestrogens in terms of both EC_{50} and E_{max} (Fig. 2). By contrast, acetylcholine-induced relaxations were almost completely abolished in the same arterial segments (Fig. 3).

The effects of the different treatments used to assess the possible involvement of NO/cGMP- and/or prostanoid-related mechanisms in the relaxant responses to the phytoestrogens, are shown in Fig. 4 and Table 2. Incubation of the arterial segments with L-NAME, ODQ, NS2028 or indomethacin did not induce significant changes in the EC_{50} values of genistein, daidzein and zearalanone, while L-NAME significantly shifted to the right (higher EC_{50} and reduced potency), the biochanin A-elicited relaxant response. The E_{max} mean value of the genistein-induced response was significantly reduced (lower efficacy) by L-NAME but not by the rest of treatments, while daidzein-, zearalanone- and biochanin A-elicited responses were unaffected by any of the treatments.

Cumulative addition of increasing concentrations of $CaCl_2$ (10^{-5}–10^{-2} M) induced concentration-dependent contractions of the arterial segments incubated in KCl-depolarized, Ca^{2+}-free medium (Fig. 5). Preincubation with phytoestrogens (10^{-5} or 10^{-4} M) significantly inhibited the $CaCl_2$-elicited contractile response in terms of both EC_{50} and E_{max} (Table 3). Zearalanone and biochanin A showed higher efficacy than genistein and daidzein in inhibiting $CaCl_2$-induced response. Biochanin A at the concentration of 10^{-4} M showed the highest inhibitory action, as it completely abolished the $CaCl_2$-induced contractile response in rabbit basilar arteries.

Table 3
Effects of phytoestrogens on $CaCl_2$-induced contractile response in rabbit-isolated basilar artery

	EC_{50} ($\times 10^{-4}$ M)	E_{max} (%)	n
Control	1.82 (1.51–2.18)	107 ± 2	38
Genistein, 10^{-5} M	2.29 (2.09–2.51)[a]	98 ± 2	14
Genistein, 10^{-4} M	5.62 (4.46–7.08)[b,c]	44 ± 5[d,e]	14
Daidzein, 10^{-5} M	2.09 (1.74–2.51)	93 ± 3[f]	12
Daidzein, 10^{-4} M	3.23 (2.57–4.07)[g]	64 ± 3[d,e]	11
Zearalanone, 10^{-5} M	7.41 (6.16–8.91)[b]	51 ± 5[d]	10
Zearalanone, 10^{-4} M	12.02 (9.55–15.1)[b,g]	28 ± 4[d,e]	12
Biochanin A, 10^{-5} M	5.37 (4.46–6.45)[b]	60 ± 3[d]	12
Biochanin A, 10^{-4} M	0[b,c]	0[d,e]	12

EC_{50} values are expressed as mean with its 95% confidence limits (in parenthesis) from "n" arterial segments. E_{max} values are expressed as percentage of previous contraction to 5×10^{-2} M KCl in the same arterial segment, and represented as mean ± S.E.M. from "n" arterial segments.
The "Control" row shows the overall mean values for EC_{50} and E_{max} from all $CaCl_2$ curves in the absence of any treatment. However, the statistical analysis were carried out by using the respective control values obtained from each experimental group.

[a] Significantly higher than Control, $P<0.05$. Student–Newman–Keuls test.

[b] Significantly higher than Control, $P<0.001$. Student–Newman–Keuls test.

[c] Significantly higher than the same phytoestrogen, $P<0.001$. Student–Newman–Keuls test.

[d] Significantly lower than Control, $P<0.001$. Student–Newman–Keuls test.

[e] Significantly lower than the same phytoestrogen, $P<0.001$. Student–Newman–Keuls test.

[f] Significantly lower than Control, $P<0.01$. Student–Newman–Keuls test.

[g] Significantly higher than the same phytoestrogen 10^{-4} M, $P<0.01$. Student–Newman–Keuls test.

4. Discussion

Increasing evidence has been accumulated in the last few years showing that phytoestrogens (particularly isoflavones) influence vascular function in several ways. However, as far as we know, the pharmacological activities of phytoestrogens on cerebral blood vessels had not yet been investigated. The first point to consider is their cerebral vasorelaxant action. Our results clearly show that acute exposure of K^+ (depolarization)- or UTP (agonist)-precontracted rabbit-isolated basilar arteries to phytoestrogens induced them to relax. These results agree with those obtained previously in other vascular beds such as rabbit coronary (Figtree et al., 2000) and rat aorta, pulmonary (Mishra et al., 2000) and mesenteric (Nevala et al., 1998) arteries. Interestingly, a direct comparison with our previous results obtained in the same experimental preparation with the natural estrogen, 17-β-estradiol (Salom et al., 2001), shows that phytoestrogens and 17-β-estradiol are equipotents in relaxing cerebral arteries. With the exception of daidzein, which relaxed more efficiently the

UTP- than the K^+-precontracted arterial segments, phytoestrogens showed a very similar relaxant profile in both conditions. Overall, it can be stated that the phytoestrogen-induced cerebral vasorelaxations are independent of the precontractile stimulus. Therefore, phytoestrogens must be interacting with some mechanism which contribution to the maintenance of cerebrovascular tone does not depend on the agent eliciting the contraction. By contrast, different relaxant potencies were shown as zearalanone was the most and daidzein the least potent relaxants. Conceivably, a structure–function relationship should exist.

The possibility has been discussed that the following mechanisms could account for the vasorelaxant effect of phytoestrogens: stimulated NO and/or prostacyclin production from endothelium, inhibition of protein tyrosine kinase (PTK), blockade of Ca^{2+} influx, activation of ATP-sensitive K^+ channels and interaction with estrogen receptors (Figtree et al., 2000; Martin et al., 2001). We focused on endothelium-related mechanisms such as the NO/cGMP pathway and prostanoid synthesis, as well as on Ca^{2+}.

The current results show that the presence of a functional endothelium is not important to the relaxant effects of phytoestrogens in rabbit basilar artery, since the responses were almost identical in intact and endothelium-denuded arteries. Similar results have been obtained in rat mesenteric (Nevala et al., 1998) and rabbit coronary (Figtree et al., 2000) arteries. Evidence against the influence of phytoestrogens on endothelial function has also been obtained in healthy, postmenopausal women receiving daily supplementation with isoflavones (Simons et al., 2000). In a previous study from our laboratory in which the same experimental preparation was used, endothelium was shown not to be important to the relaxant effects of 17-β-estradiol (Salom et al., 2001). By contrast, some studies carried out in rat aorta show that the relaxant responses to some phytoestrogens or their metabolites depend on the presence of a functional endothelium (Mishra et al., 2000; Squadrito et al., 2000; Chin-Dusting et al., 2001).

Although a significant (but modest) contribution of NO should not be discarded in the biochanin A- and genistein-elicited vasorelaxations, taken together our results show that neither NO nor vasorelaxant prostaglandins (prostacyclin) are major mediators of the phytoestrogen-elicited relaxant responses, since neither L-NAME nor indomethacin did modify such responses. Similar results have been obtained in rat mesenteric (Nevala et al., 1998) and rabbit coronary (Figtree et al., 2000) arteries. Our results extend such findings by demonstrating that increased production of cGMP, the main second messenger for relaxant responses (particularly to NO) in cerebral vessels along with cAMP (Faraci and Heistad, 1997), is not involved in the cerebral vasorelaxant responses to phytoestrogens since the potent inhibitors of guanylate cyclase, ODQ and NS2028, were without effect. By contrast, NO has been reported to mediate the genistein-elicited dilation of human forearm vasculature (Walker et al., 2001), the genistein- and daidzein-elicited relaxant responses in rat aorta (Mishra et al., 2000), and the relaxant responses elicited by some isoflavone metabolites in rat aorta (Chin-Dusting et al., 2001). Taken together our own results and those from other studies, it seems reasonable to suggest that activation of relaxant endothelium-related mechanisms (particularly the NO pathway) by phytoestrogens depends mainly on both the vascular bed and the species.

Due to its inhibitory effect on PTK, genistein has been extensively used to assess the role of protein tyrosine phosphorylation in the regulation of cerebrovascular function. Of particular interest are the observations that protein tyrosine phosphorylation is involved in the Ca^{2+}-mediated responses in cerebrovascular smooth muscle cells (Iwabuchi et al., 1999; Yang et al., 1999; Aoki et al., 2000; Kimura et al., 2000). As first noticed by Wijetunge et al. (1992) in isolated smooth muscle cells from rabbit ear artery and later confirmed in smooth muscle cells from other tissues (Kusaka and Sperelakis, 1995; Ratz et al., 1999), a direct blocking action of genistein at Ca^{2+} channels also takes place. Therefore, we analyzed the ability of the four phytoestrogens to block the Ca^{2+}-elicited contractile response in cerebral arteries as a possible mechanism explaining their relaxant effects. All four phytoestrogens blocked the Ca^{2+}-elicited contractions, which is in line with what has been previously reported by Figtree et al. (2000) in rabbit-isolated coronary arteries. Since Ca^{2+} influx is the key event leading both to maintenance of basal cerebrovascular tone and to depolarization- and agonist-induced contraction of cerebral arteries (for review, see Alborch et al., 1995), our results suggest that Ca^{2+} influx blockade could be the main mechanism involved in the phytoestrogen-induced cerebral vasorelaxation. The close correlation between the phytoestrogen concentrations relaxing K^+- or UTP-induced contraction and those blocking Ca^{2+}-induced contraction strongly supports such a suggestion. Moreover, the fact that the four phytoestrogens relaxed to the same extent the intact and the endothelium-denuded basilar artery segments demonstrates that such calcium antagonistic action takes place in the smooth muscle cells. Taking our previous results (Salom et al., 2001) to make a direct comparison, phytoestrogens are by about three orders of magnitude less potent than the dihydropyridine-type Ca^{2+} entry blocker drug nicardipine, and almost equipotent with 17-β-estradiol.

Concentrations of phytoestrogens with relaxant and calcium antagonistic effects in our experiments are in the micromolar range, and one could ask whether or not these results are relevant to the in vivo situation, particularly in humans. Phytoestrogen plasma levels have been measured in several studies in both animals and humans (for reviews, see Setchell, 1998; Bhathena and Velasquez, 2002). These studies show that phytoestrogen plasma levels are in the nanomolar range in humans consuming soy-free diets, and that the values rise to the micromolar range when soy is consumed either as an ingredient of the current diet (e.g. East Asian people, vegetarians) or as an ingredient of dietary supplements (e.g. postmenopausal women). Conceivably, phytoes-

trogens will elicit physiological effects on cerebral circulation under such situations.

In summary, the results reported here allow us to conclude that the four phytoestrogens investigated (i.e. genistein, daidzein, zearalanone and biochanin A) relaxed rabbit-isolated basilar artery by a mechanism which involves Ca^{2+} entry blockade in the smooth muscle cells rather than stimulation of vasorelaxant endothelium-related mechanisms such as the NO/cGMP pathway or prostaglandins. This occurs at concentrations which are attained when soy-based diets or soy-derived products are consumed.

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