Effect of Short-Term Phytoestrogen Treatment in Male Rats on Nitric Oxide-Mediated Responses of Carotid and Cerebral Arteries: Comparison with 17β-Estradiol

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ABSTRACT

The use of estrogen for protection against vascular dysfunction is limited due to its effects on the reproductive system, particularly in males. We postulated that daidzein, an isoflavone with estrogen-like effects on the systemic vasculature but not the reproductive system, might enhance nitric oxide (NO)-mediated cerebral vasodilatation. Male rats were administered vehicle, 17β-estradiol (0.1 mg/kg s.c.), or daidzein (0.2 mg/kg s.c.) daily for 7 days. Basal and acetylcholine-stimulated NO release was assessed in vitro via carotid arterial rings or in vivo by measuring changes in basilar artery diameter. Levels of protein expression of endothelial NO synthase (eNOS), caveolin-1, and calmodulin were assessed in carotid arteries using Western analysis. Plasma NO levels were doubled by daidzein or 17β-estradiol. NO production and endothelium-dependent contraction in response to the NOS inhibitor Nω-nitro-L-arginine (L-NNA; 100 μM) was enhanced by 50 to 100% in carotid arteries from rats treated with daidzein or 17β-estradiol. Acetylcholine-induced relaxation woke selectively enhanced in carotid arteries from rats treated with daidzein. Similarly, constrictor responses of the basilar artery to L-NNA in vivo were selectively augmented by ~100% by 17β-estradiol treatment and tended to be ~50% greater in daidzein-treated rats. Expression of caveolin-1 was decreased, and calmodulin was increased, in vessels from daidzein- or 17β-estradiol-treated rats. eNOS expression was unaffected by the treatments. These data suggest that short-term administration of daidzein or 17β-estradiol modulates cerebral artery reactivity in males by enhancing synthesis and release of endothelium-derived NO. Isoflavone therapy may therefore be a feasible approach to protect against cerebrovascular disease and stroke.

Death and morbidity from cardiovascular disease is substantially lower in premenopausal women compared with age-matched men (Barrett-Connor and Bush, 1991). After menopause, the incidence of cardiovascular mortality and morbidity, including the incidence of stroke, dramatically increases in females to levels comparable with those of males similar in age (Penotti et al., 1993). The increased incidence of vascular disease in postmenopausal women has been attributed to the absence of the hormone estrogen, specifically the predominant form, 17β-estradiol.

In experimental stroke, normal female or 17β-estradiol-treated ovariectomized rats suffer less ischemic damage than males or untreated ovariectomized rats after cerebral artery occlusion (Alkayed et al., 1998; Zhang et al., 1998; Wang et al., 1999). Furthermore, chronic 17β-estradiol treatment enhances acetylcholine-induced cerebral vasodilatation (Pelligrino et al., 2000; Watanabe et al., 2001) in ovariectomized female rats. A major mechanism of the beneficial effects of 17β-estradiol is thought to involve increased expression and/or activity of endothelial nitric oxide synthase (eNOS) and consequently increased synthesis of endothelium-derived NO (McNeill et al., 1999).

Nevertheless, it is presently uncertain to what extent, if any, hormone replacement therapy (HRT) with estrogen may be beneficial in preventing clinical heart disease and stroke. More than 30 observational studies have reported up to 50% reduced risk of cardiovascular disease among postmenopausal women who use HRT (Hurn and Brass, 2003). However, in the Heart and Estrogen-Progestin Replacement Study, the first prospective, double-blind, randomized, placebo-controlled trial of HRT in postmenopausal women with known coronary heart disease, HRT did not reduce risk of stroke (Simon et al., 2001; Hurn and Brass, 2003). Moreover,
with regard to cerebrovascular health, postmenopausal estrogen use has been associated with either increased or decreased risk of stroke, or no net benefit for stroke prevention (Baron et al., 1998; Simon et al., 2001; Beral et al., 2002; Rossouw et al., 2002; Hurn and Brass, 2003).

Regardless of any potential protective actions of 17β-estradiol on cerebral and peripheral vasculature, this agent causes feminization in men and increased incidence of breast cancer and endometrial hyperplasia in women. Thus, it is desirable that therapies be identified that mimic the vaso-protective actions of 17β-estradiol without affecting the reproductive system and other nonvascular tissue (Lissin and Cooke, 2000). This goal has led to the increased study of phytoestrogens, plant-derived compounds such as daidzein, a major isoflavone mainly derived from soybean, which seems to have estrogen-like activity on the cardiovascular system without effects on the reproductive system or on tyrosine kinase activity (Lissin and Cooke, 2000; Belcher and Zsarnovszky, 2001; Mitchell et al., 2001). Like 17β-estradiol, chronic treatment with isoflavones may enhance vascular NO synthesis or activity (Squadrito et al., 2000). However, the effects of isoflavones on cerebral vascular function are largely unknown. The major aim of this study was therefore to determine the effect of short-term treatment with daidzein on cerebral vascular reactivity in vitro and in vivo. Because of the effects daidzein shares with 17β-estradiol in the peripheral vasculature, we also were interested to compare the effects of both agents in the cerebral circulation. We chose to administer daidzein subcutaneously in light of an emerging concept that intestinal metabolism of isoflavones by bacterial flora of the gut produces metabolites that may contribute to the vascular actions of isoflavones (Setchell et al., 2002). Importantly, at least in humans, there seems to exist significant individual variation in the presence of such bacterial flora (Setchell et al., 2002). Thus, subcutaneous administration avoids the functional effects of potential variation in such gastrointestinal metabolism.

Materials and Methods

General Procedures. Male Sprague-Dawley rats were studied (250–300 g; n = 75). The study was approved by the Institution’s Animal Experimentation Ethics Committee. Rats were treated with daily subcutaneous injections of vehicle (10% dimethyl sulfoxide/90% saline) or 17β-estradiol (0.2 mg/kg), or 17β-estradiol (0.1 mg/kg) for 7 days, and then killed by exposure to 80% CO2/20% O2. The dose of 17β-estradiol has previously been shown to improve endothelial function within normal levels (pH of 7.39 ± 0.01; pCO2 of 35 ± 4 mm Hg; and pO2 of 128 ± 3 mm Hg). The cranial window was continuously suffused at 2 ml/min with artificial cerebrospinal fluid. Cerebrospinal fluid sampled from the cranial window was pCO2 of 7.41 ± 0.02, pCO2 of 35 ± 4 mm Hg; and pO2 of 128 ± 3 mm Hg. Diameter of the basilar artery was monitored using a microscope coupled to a video monitor and was continuously measured using a computer-based tracking program (Diamtrak; Montech Clayton, Australia). After the craniotomy was prepared, the artery was allowed 30 min to equilibrate before application of vasoconstrictors, which were applied topically. Serotonin (0.1 μM) was applied until a steady-state change in diameter was attained, which usually occurred within 5 to 6 min. The basilar artery was then allowed to recover for at least 30 min. To determine the effect of short-term daidzein or 17β-estradiol on basal NO activity in the basilar artery, l-Arginine (100 μM) was similarly applied topically for 30 min.

Western Analysis of NOS, Caveolin-1, and Calmodulin. Sections of carotid arteries that were not used in functional experiments were precontracted to 50% of the KPSS response using phenylephrine, and concentration-response curves were performed for both agents in the cerebral circulation. We chose to determine the effect of short-term treatment with daidzein or 17β-estradiol on basal NO activity. First, we measured the level of NO metabolite byproducts (NOx, i.e., nitrite + nitrate) in plasma, and their production was measured in samples of plasma or culture medium after converting all nitrate to nitrite with nitrate reductase. Total nitrite levels were measured in duplicate by absorbance at 560 nm using the Griess reagent of 50% (N-1-naphthyl)ethylenediamine hydrochloride/50% sulfanilamide in 100 ml of 5% (v/v) orthophosphoric acid added to 100 ml of medium. Sodium nitrite was used as a standard. Second, contraction in response to the isofomselective NOS inhibitor Nω-nitro-l-arginine (l-NNA; 100 μM) was measured. Endothelium-intact and endothelium-denuded rings were precontracted to ~20% of the contraction to KPSS using phenylephrine, and l-NNA was added to the tissue once a stable level of contraction was obtained. Arterial rings were allowed to reach maximum contraction (~30 min). The response to l-NNA was expressed as a percentage of the contraction to KPSS (not including the precontracted tone).

Effect of Short-Term Treatment with Daidzein or 17β-Estradiol on Basal NO Activity. We used two approaches to assess the effect of short-term treatment with daidzein or 17β-estradiol on basal NO activity. First, we measured the level of NO metabolic byproducts (NOx, i.e., nitrite + nitrate) in plasma, and their production was measured in samples of plasma or culture medium after converting all nitrate to nitrite with nitrate reductase. Total nitrite levels were measured in duplicate by absorbance at 560 nm using the Griess reagent of 50% (N-1-naphthyl)ethylenediamine hydrochloride/50% sulfanilamide in 100 ml of 5% (v/v) orthophosphoric acid added to 100 ml of medium. Sodium nitrite was used as a standard. Second, contraction in response to the isofomselective NOS inhibitor Nω-nitro-l-arginine (l-NNA; 100 μM) was measured. Endothelium-intact and endothelium-denuded rings were precontracted to ~20% of the contraction to KPSS using phenylephrine, and l-NNA was added to the tissue once a stable level of contraction was obtained. Arterial rings were allowed to reach maximum contraction (~30 min). The response to l-NNA was expressed as a percentage of the contraction to KPSS (not including the precontracted tone).
were frozen, homogenized, and subsequently used for Western analysis. Briefly, identical amounts of protein were separated on 7.5, 12, and 15% SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose, with a Trans-blot (Bio-Rad, Hercules, CA) overnight. Blots were blocked with 5% (w/v) skim milk in phosphate-buffered saline (PBS) for 1 h at room temperature. Blots were then incubated overnight at 4°C with the primary antibodies, anti-eNOS, anti-caveolin-1 (mAb 1:1000; BD Transduction Laboratories, Lexington, KY) and anti-calmodulin (mAb 1:1000; Auspep, Melbourne, Australia) diluted in 3% bovine serum albumin/PBS. Membranes were then incubated with the sheep anti-mouse secondary antibody (1:1000; Silenus, Boronia, Australia) diluted in 3% bovine serum albumin/PBS for 1 h at room temperature followed by five washes with 0.1% Tween in PBS. The specific bands were detected using enhanced chemiluminescence reagents and opposed to film (Amersham Biosciences, Piscataway, NJ) before development (X-ray developer and fixer; Ilford, Cheshire, UK). Protein bands detected were quantified by densitometry (Kodak Image Station 440CF; PerkinElmer Life and Analytical Sciences, Boston, MA).

**Drugs.** All drugs were purchased from Sigma-Aldrich (St. Louis, MO), except acetylcholine perchlorate (BDH, Poole, Dorset, UK), daidzein (4',7-dihydroxyisoflavone; Indofine, Hillsborough, NJ), U46619 (9,11-dideoxy-9α,11α-epoxymethano-prostaglandin F₂α; Cayman Chemical, Ann Arbor, MI), and phenoxbenzamine HCl (MP Biomedicals, Irvine, CA). Daidzein and 17β-estradiol were dissolved in 10% dimethyl sulfoxide (vehicle). With the exception of nifedipine, all other drugs were dissolved in distilled water. Analyses and Statistics. Concentration-response data were fit to a sigmoid plot using GraphPad Prism version 3.0a (GraphPad Software Inc., San Diego, CA), which estimated the pEC₅₀ value. Relaxation responses are presented as percentage of inhibition of the precontracted level of tone. Each n represents the number of animals per group. Statistical analysis was carried out using Student's unpaired t test or a one-way ANOVA followed by Dunnett's multiple comparisons test (as appropriate). P < 0.05 was considered statistically significant. All values are mean ± S.E.M.

**Results**

In comparison with vehicle-treated rats, plasma NOₓ levels were approximately doubled in rats treated with either daidzein or 17β-estradiol (Fig. 1a; both P < 0.05). Similarly, NOₓ production by carotid arteries from daidzein- or 17β-estradiol-treated rats was also ~2-fold greater than the amount produced by vessels from vehicle-treated rats (Fig. 1b; both P < 0.05). During the 7-day treatment period, the body weight gained by vehicle- and daidzein-treated rats was similar (20 ± 1 versus 21 ± 2 g; P > 0.05), whereas 17β-estradiol-treated rats gained slightly less weight (16 ± 1 g; P < 0.05 versus vehicle-treated rats). However, there was no significant effect of the treatments on testicular weight (in grams per kilogram of body weight) vehicle-treated, 7.3 ± 0.6; daidzein-treated, 8.9 ± 0.6; and 17β-estradiol-treated, 10.1 ± 0.6).

Acetylcholine and sodium nitroprusside each induced concentration-dependent relaxation of carotid artery rings. These responses were similar in vehicle-, daidzein-, and 17β-estradiol-treated rats (Table 1). To simulate responses in the presence of impaired endothelial function, acetylcholine-induced relaxation also was assessed in the presence of PBZ. In comparison with responses of carotid arteries from vehicle-treated rats, acetylcholine caused greater relaxation of arteries from rats treated with daidzein (Fig. 2, P < 0.05; Table 1). Acetylcholine-induced relaxation of rings from 17β-estradiol-treated rats also tended to be greater than those from vehicle-treated rats, but this difference did not reach statistical significance (Fig. 2, Table 1).

In endothelium-intact carotid artery rings precontracted to 20% of the KPSS-induced level using phenylephrine, contractions by L-NNA were significantly greater in vessels from rats treated with either daidzein or 17β-estradiol (Fig. 3). L-NNA had no significant effect on contractile tone in endothelium-denuded arterial rings (data not shown).

In cranial window preparations, L-NNA (100 μM) caused marked vasoconstriction in vivo in all three groups of rats (Fig. 4a). However, vasoconstrictor responses to L-NNA were approximately 50% greater in daidzein-treated rats (P > 0.05) and 100% greater in 17β-estradiol-treated rats compared with responses in vehicle-treated rats (P < 0.05; Fig. 4a). By contrast, serotonin caused equivalent vasoconstrictor responses in all three groups (Fig. 4b).

Molecular expression of eNOS was similar in carotid arteries from all three treatment groups (Fig. 5a). Expression of caveolin-1 was ~50% lower in both daidzein-treated and 17β-estradiol-treated arteries than in arteries from vehicle-treated rats (Fig. 5b). Expression of calmodulin was increased by 60–70% in daidzein-treated and 17β-estradiol-treated arteries (Fig. 5c).

**Discussion**

This is the first study to investigate effects of short-term phytoestrogen treatment on vascular responses, expression of NO-related proteins or generation of NO in cerebral arteries. Our findings indicate that, similar to the effects of 17β-
estradiol treatment, administration of daidzein for 7 days enhances NO production and consequent vasodilatation in cerebral arteries. Evidence supporting this conclusion is that production of NO metabolites, vasoconstriction by L-NNA, and vasorelaxation by acetylcholine are all augmented in carotid arteries of rats treated with either daidzein or 17β-estradiol in comparison with arteries from vehicle-treated rats. Further findings suggested that basal NO activity in the basilar artery in vivo was also higher in daidzein- and 17β-estradiol-treated rats. These effects are not due to increased expression of eNOS protein directly, but they are associated with altered levels of two eNOS-modulating proteins, specifically, decreased expression of caveolin-1 and increased expression of calmodulin.

Treatment with daidzein or 17β-estradiol for 7 days enhanced contraction in response to the NOS inhibitor L-NNA both in vitro and in vivo. Because this indicated an increase in basal NO activity, it was somewhat surprising that the relaxant response to acetylcholine was unaffected by either treatment. We considered two possible explanations for the apparently divergent results. Could daidzein or 17β-estradiol treatment selectively enhance basal rather than stimulated NO activity? Alternatively, is stimulated NO activity near-maximal under normal conditions, and thus there is little or no capacity within the system for further increase? To address the second possibility, we acutely exposed the isolated carotid arteries to PBZ to alkylate some of the muscarinic acetylcholine receptors. We found that acetylcholine-induced relaxation was not affected by PBZ treatment. However, the relaxation response to a NO donor, sodium nitroprusside (SNP), was significantly reduced by PBZ treatment in daidzein- and 17β-estradiol-treated rats but not in vehicle-treated rats. These results suggest that the enhanced basal NO activity observed in daidzein- and 17β-estradiol-treated rats is not due to increased basal NO production but rather to altered NOS activity.

### Table 1

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Vehicle</th>
<th>Daidzein</th>
<th>E2</th>
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<tbody>
<tr>
<td>ACh</td>
<td>n=7</td>
<td>7.66 ± 0.24</td>
<td>97 ± 1</td>
</tr>
<tr>
<td>ACh after PBZ</td>
<td>n=5</td>
<td>5.94 ± 0.07</td>
<td>48 ± 7</td>
</tr>
<tr>
<td>SNP</td>
<td>n=6</td>
<td>8.66 ± 0.08</td>
<td>102 ± 1</td>
</tr>
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* P < 0.05, Dunnett’s test compared with vehicle.
compared to Western societies, and reports that Japanese women suffer significantly fewer menopausal symptoms (Nestel et al., 1999) may be associated with the larger consumption of isoflavones in Asian diets compared with Western diets. Indeed, soy isoflavones are known to enhance endothelium-dependent vasodilatation in atherosclerotic monkeys (Honore et al., 1997) and to enhance aortic endothelial function in ovariectomized rats (Squadrito et al., 2000). Isoflavones also acutely dilate human forearm vasculature (Walker et al., 2001) and enhance systemic vascular compliance (Walker et al., 2001). In healthy age-matched men and postmenopausal women, consumption of a soy protein isolate diminished after the onset of menopause (Hurn and Macrae, 1999). Experimental 17β-estradiol reduced testicular volume, or semen parameters (Mitchell et al., 2001). Moreover, other significant impediments to the therapeutic use of estrogen include the development of breast and endometrial cancers in women and unacceptable effects on reproductive function in men. Hence, development of a vascular-selective alternative to estrogen therapy is clearly desirable, and this goal has led to considerable interest in the actions of plant-derived estrogen mimetics, such as the isoflavones.

Vascular Effects of Isoflavones. In the present study, and in others where a direct comparison has been made, the vascular effects of isoflavones mimic those of 17β-estradiol (Squadrito et al., 2000; Belcher and Zsarnovszky, 2001; Walker et al., 2001). Isoflavones such as genistein and daidzein are abundant in some foods, particularly soybean and legumes. It has been postulated that the relatively lower incidence of cardiovascular disease in Asian populations compared to Western societies, and reports that Japanese women

receptors and hence reduced both the potency and efficacy of acetylcholine (Martin et al., 1992). We then found that in the presence of PBZ, the relaxant responses to acetylcholine were enhanced by short-term treatment with either daidzein or 17β-estradiol. Thus, the ability of daidzein and 17β-estradiol to enhance endothelial function is most apparent when there is an impairment of stimulated NO release, emphasizing the potential for isoflavones to enhance endothelium-dependent vasorelaxation impaired by vascular disease. At present, we also cannot exclude the possibility that an up-regulation of endothelium-derived hyperpolarizing factor activity contributed to this enhancement of the vasorelaxant response to acetylcholine (Woodman and Boujaoude, 2004).

Therapeutic Use of Estrogen. The risk of cardiovascular disease, including stroke, is lower in premenopausal women than in age-matched men, but this difference progressively diminishes after the onset of menopause (Hurn and Macrae, 2000; Hurn and Brass, 2003). These different, gender-related profiles in the clinical incidence of cardiovascular disease are thought to be due to differences, or changes, in the plasma level of estrogen. However, despite substantial experimental evidence that estrogen can improve vascular function, recent large clinical trials investigating the effects of HRT, using estrogen plus progesterone or estrogen alone, report no benefit on the incidence of coronary artery disease or stroke (Simon et al., 2001; Beral et al., 2002; Grady et al., 2002; Hurn and Brass, 2003). Moreover, other significant impediments to the therapeutic use of estrogen include the development of breast and endometrial cancers in women and unacceptable effects on reproductive function in men. Hence, development of a vascular-selective alternative to estrogen therapy is clearly desirable, and this goal has led to considerable interest in the actions of plant-derived estrogen mimetics, such as the isoflavones.

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suffer significantly fewer menopausal symptoms (Nestel et al., 1999) may be associated with the larger consumption of isoflavones in Asian diets compared with Western diets. Indeed, soy isoflavones are known to enhance endothelium-dependent coronary vasodilatation in atherosclerotic monkeys (Honore et al., 1997) and to enhance aortic endothelial function in ovariectomized rats (Squadrito et al., 2000). Isoflavones also acutely dilate human forearm vasculature (Walker et al., 2001) and enhance systemic vascular compliance (Walker et al., 2001). In healthy age-matched men and postmenopausal women, consumption of a soy protein isolate was reported to decrease arterial pressure (Teede et al., 2001). In contrast to endogenous estrogens, however, isoflavones do not seem to affect reproductive function and are more selective for vascular and other nonreproductive tissues (Lissin and Cooke, 2000; Belcher and Zsarnovszky, 2001). We found that neither daidzein nor 17β-estradiol reduced testicular weight, thus the vascular effects of these treatments did not seem to be associated with inhibitory effects on the reproductive system during this short-term study. Similarly, a recent study in humans has found that in male volunteers, 2 months of dietary phytoestrogen supplementation high in daidzein also had no effect on endocrine measurements, testicular volume, or semen parameters (Mitchell et al., 2001).

Estrogen-Like Effects of Daidzein on Expression of NOS-Related Proteins. A major protective effect of estrogen against vascular disease may occur via increasing the bioactivity of vascular NO (i.e., by increasing NO synthesis and/or stability). NOS enzymes are subject to transcriptional and post-translational regulation, and there is evidence that estrogen affects both of these processes (Papapetropoulos et al., 1999). Experimental 17β-estradiol treatment has variously been reported to increase expression of each of the three NOS isoforms (Binko and Majewski, 1998; Pelligrino et al., 1998, 2000; McNeill et al., 1999; McNeill et al., 2002; Mershon et al., 2002). By contrast, 17β-estradiol may increase NO synthesis in aorta of male rats (M. Boujaoude and O. L. Woodman, unpublished observations) and of ovariectomized mice (Darblade et al., 2002) without changing expression of any NOS isoform. Although NO production and activity was also higher in the carotid artery after daidzein or 17β-estradiol treatment in the present experiments, we found that levels of eNOS expression also were not altered in
this vessel. Because 1-NNa had no constrictor effect in endothelium-denuded arteries, it seems that the greater levels of basal NO release after daidzein or 17β-estradiol treatment were not due to upregulation of expression of inducible NOS in vascular smooth muscle (Binko and Majewski, 1998; Mershon et al., 2002).

Two proteins that are also critical in the regulation of eNOS activity are caveolin-1 (inhibitory) and calmodulin (activating), which compete for the same binding domain on eNOS. Altered expression of these modulatory proteins could have profound effects on vascular NO synthesis and contractile tone. It has been reported that 17β-estradiol treatment reverses the increase in caveolin-1 expression in female rat pial arterioles that occurs after ovariectomy (Pelligrino et al., 2000). Two important novel findings of the present study are that short-term treatment with either daidzein or 17β-estradiol decreases caveolin-1 expression and increases calmodulin expression in carotid arteries of normal males. Such findings support the hypothesis that, similar to effects of estrogen, post-translational modulation of NOS activity via altered expression of eNOS modulatory proteins may be an important target for isoflavones to increase NO activity in the cerebral circulation.

Although estrogen and the isoflavones can exert rapid, acute effects directly on vascular tone, it is likely that the actions of 17β-estradiol and daidzein observed here were mainly genomic, because remarkably similar degrees of altered caveolin-1 and calmodulin protein expression due to the modulation of gene transcription were observed. In further support of this proposition is the fact that daidzein and 17β-estradiol have similar transactivation potentials at an estrogen responsive element, whereas daidzein has 1000 and 200 times less binding affinity than 17β-estradiol at the estrogen receptors ERα and ERβ, respectively (Belcher and Zsarnovszky, 2001).

In summary, the results of the present study demonstrate that short-term treatment with daidzein mimics the effect of 17β-estradiol in augmenting NO synthesis and activity in carotid and basilar arteries of male rats. These effects are associated with no change in eNOS expression but with increased calmodulin and decreased caveolin-1 expression in the carotid arteries. Thus, estrogen-like effects in the cerebral circulation can be achieved using isoflavones, which increases activity of endothelium-derived NO, probably via altered expression of key proteins which modulate eNOS activity.

References
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