The Phytoestrogen Genistein Produces Acute Nitric Oxide–Dependent Dilation of Human Forearm Vasculature With Similar Potency to 17β-Estradiol

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Background—Genistein, a phytoestrogen, may have estrogenic cardioprotective actions. We investigated whether genistein influences endothelium-dependent vasodilation in forearm vasculature of healthy human subjects and compared the effects of genistein with those of 17β-estradiol.

Methods and Results—The brachial arterial was cannulated with a 27-gauge needle for drug infusion. Forearm blood flow responses were measured with strain-gauge plethysmography. Genistein (10 to 300 nmol/min, each dose for 6 minutes) produced a dose-dependent increase in forearm blood flow from 3.4±0.3 to 9.6±1.3 mL · min^{-1} · 100 mL forearm^{-1} (mean±SEM) in men (n=9, $P<0.0001$ by ANOVA). The mean forearm venous concentration of genistein during infusion of the highest dose was 1.8±0.3 μmol/L in 6 additional men. Genistein produced a similar increase in blood flow in premenopausal women. Daidzein, another phytoestrogen, was ineffective, but equimolar concentrations of 17β-estradiol caused similar vasodilation to genistein. Responses to genistein and 17β-estradiol were inhibited to the same degree by the NO synthase inhibitor N^G-monomethyl-L-arginine. A threshold dose of genistein potentiated the endothelium-dependent vasodilator acetylcholine but not the endothelium-independent vasodilator nitroprusside.

Conclusions—Genistein causes L-arginine/NO-dependent vasodilation in forearm vasculature of human subjects with similar potency to 17β-estradiol and potentiates endothelium-dependent vasodilation to acetylcholine. **(*Circulation*. 2001;103:258-262.)**

Key Words: genistein ■ vasodilation ■ hormones ■ endothelium ■ nitric oxide

Estrogen has protective effects on the cardiovascular system.[1] Genistein, a phytoestrogen, may have estrogenic cardioprotective actions[2] and enhances coronary vasoreactivity in macaque monkeys.[3] The affinity of genistein for the classic estrogen receptor (ER)-α present on reproductive organs is less than that of estrogen.[4] However, genistein has a similar affinity as estrogen for the novel ER-β present in the vasculature. Genistein can be administered to both men and women without causing conventional estrogenic effects. Indeed, genistein is present in high concentrations in the diet of East Asian subjects,[5] and it is possible that high plasma concentrations of genistein contribute to the strikingly low incidence of atherosclerosis and coronary heart disease (CHD) seen in East Asia.[2,6]

We performed pilot studies to assess the ability of genistein to enhance endothelium-dependent vasodilation in human forearm vasculature. To our surprise, we found that the brachial artery infusion of genistein produced marked vasodilation of forearm resistance vasculature at concentrations that at threshold did not greatly exceed those reported in plasma in East Asian subjects (maximum concentrations were ≈10-fold higher). Such responses rapidly reached a plateau and returned to baseline within a few minutes. We therefore performed further studies to examine the mechanism by which genistein causes vasodilation. We examined forearm blood flow responses to brachial artery infusion of genistein in men and women in the presence and absence of N^G-monomethyl-L-arginine (L-NMMA), an inhibitor of NO synthase. We compared the response to genistein with that to 17β-estradiol and to another phytoestrogen, daidzein, which has a low affinity for both ER-α and ER-β.[4] In addition, we examined the ability of threshold concentrations of genistein to augment the vasodilator effects of the endothelium-dependent vasodilator acetylcholine.

Methods

Subjects

Forearm blood flow studies were performed in healthy men aged 20 to 51 years and healthy premenopausal women aged 29 to 33 years. No subject was receiving drug therapy (including contraceptives).

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Arterial blood pressure was 120±8/74±7 mm Hg in men and 117±5/73±6 mm Hg in women (mean±SD). Mean serum cholesterol levels were 4.5±1.0 and 4.8±0.4 mmol/L in men and women, respectively. The study was approved by the St Thomas' Hospital Research Ethics Committee, and all subjects gave written informed consent.

Forearm Blood Flow

Forearm blood flow measurements were made in a quiet clinical laboratory (temperature controlled to between 24° and 26°C during each study). Blood flow was measured in both forearms with venous occlusion plethysmography with strain gauges[7] that were electrically calibrated.[8] Wrist occlusion pressure was 180 mm Hg, and collecting cuff pressure was 40 mm Hg. An unmounted 27-gauge steel needle (Cooper's Needleworks) was sealed with dental wax to an epidural catheter and inserted into the left brachial artery under sterile conditions with <1 mL of 1% lignocaine hydrochloride to provide local anesthesia. After cannulation of the brachial artery, saline was infused at 1 mL/min for 30 minutes to establish baseline flow with a constant-rate infusion pump. Drugs or saline were then infused at 1 mL/min according to the protocols given later. Forearm blood flow was recorded during the final 3 minutes of each infusion period with 10-second venous occlusions every 15 seconds. Blood flow was calculated from the mean values of the last 5 venous inflations and expressed in $mL \cdot min^{-1} \cdot 100$ mL of forearm $volume^{-1}$.[7] Blood pressure was measured with mercury sphygmomanometry at the end of the study.

Forearm Blood Flow Responses to Genistein, Daidzein, and Vehicle

The effects of genistein were studied in men (n=9) and women (n=6). Women were studied between days 10 and 14 of the menstrual cycle. After baseline blood flow was established, subjects received a cumulative rising-dose brachial artery infusion of genistein (10, 30, 100, and 300 nmol/min, each dose for 6 minutes). On separate occasions, additional men received an infusion of daidzein (10, 30, 100, and 300 nmol/min, each dose for 6 minutes; n=6) and genistein/daidzein vehicle (n=6) alone. In an additional study in 6 men with the same protocol, plasma concentrations of genistein in the antecubital vein of the infused forearm were measured at baseline and during the final infusion of genistein. Plasma genistein concentration was determined with a modification of the method of Adlercreutz et al[9] with gas chromatography–electron impact mass spectroscopy and tetradeuterated genistein used as internal standard.

Effects of L-NMMA on the Blood Flow Response to Genistein

Two protocols were used to assess the effects of L-NMMA on the response to genistein. Each protocol consisted of studies performed on 2 days separated by 1 week. In protocol 1, 6 men received an infusion of genistein followed, after a washout period, by L-NMMA on 1 occasion and saline on the other and then a second infusion of genistein. On each day, after the establishment of baseline flow, genistein (10, 30, 100, and 300 nmol/min, each dose for 6 minutes) was coinfused with saline. Saline was then infused alone for 20 minutes until blood flow returned to baseline. L-NMMA (8 μmol/min) on 1 occasion and saline placebo on the other occasion were infused alone for 6 minutes and then coinfused with a second identical rising-dose infusion of genistein. Protocol 2 was used to exclude the effects of baseline drift and of any carryover effect related to sequential infusions of genistein during the course of a single study. Such carryover effects might include an alteration in basal NO synthase activity by the first infusion of genistein. Men (n=9) were studied on 2 occasions. After the establishment of baseline flow, L-NMMA (8 μmol/min) on 1 occasion and saline placebo on the other occasion were infused alone for 6 minutes and then coinfused during a single rising-dose infusion of genistein (10, 30, 100, and 300 nmol/min, each dose for 6 minutes).

In 6 subjects, the effects of L-NMMA on responses to nitroprusside, a short-acting endothelium-independent vasodilator, were studied as a control with a study design similar to that of protocol 1, as given. After the infusion of saline to establish baseline flow, subjects received a cumulative rising-dose infusion of nitroprusside (1, 3, and 10 nmol/min, each for 6 minutes). After the return of blood flow to baseline, L-NMMA (8 μmol/min) was infused alone for 6 minutes and then coinfused during a second rising-dose infusion of nitroprusside.

Effects of 17β-Estradiol With and Without L-NMMA

After the establishment of baseline flow, men (n=5) received 17β-estradiol (10, 30, and 100 nmol/min, each dose for 6 minutes) coinfused with saline. Saline was then infused alone for 20 minutes until blood flow returned to baseline. L-NMMA (8 μmol/min) was then infused alone for 6 minutes and then coinfused with a second identical rising-dose infusion of estrogen.

Effects of a Threshold Dose of Genistein on Vasodilator Responses to Acetylcholine and Nitroprusside

The effect of genistein at a dose of 30 nmol/min, which when infused alone produced no significant increase in forearm blood flow, on vasodilator responses to acetylcholine and nitroprusside was determined in 6 men. After baseline blood flow was established, subjects received a rising-dose brachial artery infusion of acetylcholine (20, 40, and 80 nmol/min, each dose for 6 minutes). After a 20-minute recovery period, genistein (30 nmol/min) was infused alone for 20 minutes and then throughout a second cumulative infusion of acetylcholine as before. This protocol was repeated in an additional group of 6 men with a cumulative rising-dose brachial artery infusion of nitroprusside (1, 3, and 10 nmol/min, each for 6 minutes) instead of acetylcholine.

Drugs

Genistein, estrogen, and daidzein (Clinalfa) were lyophilized with glycocholate/lecithin to obtain a water-soluble micellar preparation. L-NMMA, acetylcholine, and sodium nitroprusside were obtained from Clinalfa, Coopervision, and Roche, respectively. All drugs were diluted in saline (0.9% NaCl).

Statistical Analysis

Results are presented as mean±SEM. Vasodilator responses were expressed as the increase above the immediately preceding baseline.[10] For the comparison of responses to genistein and estrogen, responses were summarized as the area under the dose-response curve (AUC). Differences in responses were sought with ANOVA for repeated measures. Differences were considered to be significant at a value of $P<0.05$ (2-sided).

Results

Blood flow in the noninfused (control) arm did not change significantly during any study day.

Vasodilator Effects of Genistein and Daidzein in Men and Women

Genistein/daidzein vehicle produced no significant change in forearm blood flow (Figure 1). Daidzein (10 to 300 nmol/min) also produced no significant change in forearm blood flow, whereas genistein increased blood flow. During infusion of the highest dose (300 nmol/min), blood flow increased from 3.4±0.3 to 9.6±1.3 $mL \cdot min^{-1} \cdot 100$ mL^{-1} in men ($P<0.0001$, Figure 1) and from 3.3±0.6 to 7.5±0.7 $mL \cdot min^{-1} \cdot 100$ mL^{-1} in women ($P=0.0001$, Figure 1). Plasma concentrations of genistein during infusion of the

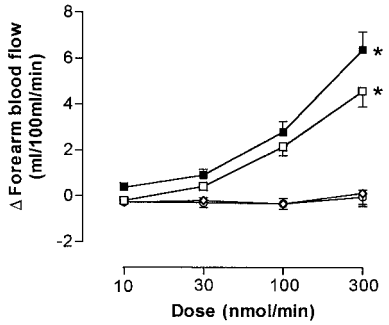

Figure 1. Increase above baseline in forearm blood flow (Δforearm blood flow) after brachial artery infusion of genistein/daidzein vehicle (○, n=6), daidzein (◇, n=6), and genistein in men (■, n=9) and premenopausal women (□, n=6). *$P<0.0001$ compared with vehicle by ANOVA for all doses.

highest dose were estimated from the rates of arterial drug infusion and forearm blood flow and were in the range of 1 to 10 μmol/L. In further experiments in which plasma genistein was measured directly, the mean concentration of genistein in antecubital veins draining the infused forearm during infusion of the highest dose was 1.8±0.3 μmol/L (n=6).

Effects of L-NMMA on the Vasodilator Response to Genistein

The vasodilator response to genistein was blunted in the presence of L-NMMA as assessed with the use of protocol 1. The highest dose of genistein (300 nmol/min) increased forearm blood flow from 3.7±0.3 to 9.5±2.0 $mL \cdot min^{-1} \cdot 100\ mL^{-1}$ during coinfusion with saline. Blood flow returned to 4.6±1.9 $mL \cdot min^{-1} \cdot 100\ mL^{-1}$ after a 20-minute washout period, decreased to 2.9±0.9 $mL \cdot min^{-1} \cdot 100\ mL^{-1}$ during the infusion of L-NMMA, and then increased to 6.0±0.6 $mL \cdot min^{-1} \cdot 100\ mL^{-1}$ during the subsequent coinfusion of genistein with L-NMMA ($P<0.002$ for the comparison of L-NMMA versus saline; Figure 2). In the control study performed on a separate occasion, a second infusion of genistein with saline placebo instead of L-NMMA produced similar increases in blood flow in response to the first infusion of genistein. Similar results were obtained when the effects of L-NMMA were assessed with protocol 2. During coinfusion with saline, genistein (300 nmol/min) increased forearm blood flow from 3.4±0.3 to 9.6±1.3 $mL \cdot min^{-1} \cdot 100\ mL^{-1}$. On the other study day, L-NMMA decreased blood flow from 4.2±0.5 to 3.1±0.4 $mL \cdot min^{-1} \cdot 100\ mL^{-1}$, and during coinfusion with L-NMMA, genistein (300 nmol/min) increased blood flow to 5.7±0.5 $mL \cdot min^{-1} \cdot 100\ mL^{-1}$ ($P<0.001$ for the comparison with saline). L-NMMA had no significant effect on the blood flow response to nitroprusside. Nitroprusside (10 nmol/min) increased forearm blood flow from 3.4±0.86 to 12.1±2.3 $mL \cdot min^{-1} \cdot 100\ mL^{-1}$ during coinfusion of saline compared with an increase from 2.5±0.45 to 11.7±2.2 $mL \cdot min^{-1} \cdot 100\ mL^{-1}$ during coinfusion with L-NMMA (P=NS).

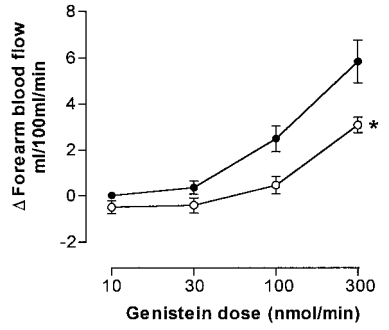

Figure 2. Increase above baseline in forearm blood flow (Δforearm blood flow) during coinfusion of genistein with saline (●) and then after coinfusion with L-NMMA (8 μmol/min, ○) on the same occasion (n=6). *$P<0.002$ for comparison of genistein plus saline vs genistein plus L-NMMA by ANOVA for all doses.

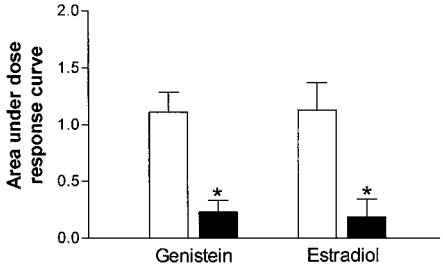

Figure 3. Vasodilator effects of genistein and 17β-estradiol (AUC) during coinfusion of saline and L-NMMA (8 μmol/min, n=5). *$P<0.01$ for L-NMMA vs saline.

Comparison of Effects of Genistein With Those of 17β-Estradiol

Equimolar concentrations of genistein and 17β-estradiol produced similar vasodilator effects, and the inhibitory effect of L-NMMA on the response to estrogen was similar to that to genistein (Figure 3).

Effects of a Threshold Dose of Genistein on Vasodilator Responses to Acetylcholine and Nitroprusside

The infusion of genistein (30 nmol/min) had no significant effect on blood flow but, during coinfusion with acetylcholine, potentiated the response to acetylcholine compared with that obtained during coinfusion with saline (Figure 4). The highest dose of acetylcholine (80 nmol/min) increased blood

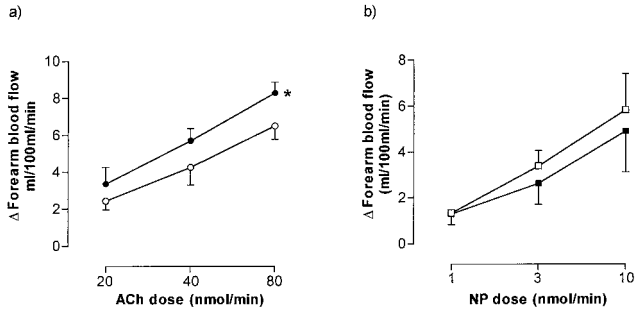

Figure 4. a, Increase above baseline in forearm blood flow (Δforearm blood flow) during coinfusion of acetylcholine with saline (○) and near-threshold dose of genistein (30 nmol/min, ●, n=6). *$P<0.01$ for comparison of acetylcholine plus genistein vs acetylcholine plus saline by ANOVA for all doses. b, Increase above baseline in forearm blood flow (Δforearm blood flow) during coinfusion of nitroprusside with saline (□) and genistein (30 nmol/min, ■, n=6, P=NS).

flow from 4.6±0.9 to 11.1±1.4 mL · min^{-1} · 100 mL^{-1} during coinfusion with saline and from 5.3±0.9 to 13.5±1.1 mL · min^{-1} · 100 mL^{-1} during coinfusion with genistein ($P<0.01$ for the comparison). The same dose of genistein had no significant effect on the response to nitroprusside (Figure 4).

Discussion

The first novel finding of the present study is that acute brachial artery administration of genistein produces vasodilation in forearm vasculature. The degree of vasodilation, which is comparable to that achieved with pharmacological concentrations of the vasodilators acetylcholine and nitroprusside, is surprising. A 2- to 3-fold increase in blood flow is caused by dosages that produce forearm venous plasma concentrations of ≈2 μmol/L. Plasma concentrations of genistein of ≈0.3 μmol/L have been observed in subjects eating a diet high in soy protein.[5] The marked vasodilation observed in this study was thus achieved at concentrations ≈10-fold higher than "physiological" concentrations. Threshold effects were, however, observed at lower doses, corresponding to concentrations not greatly exceeding those found in East Asian subjects.[5]

Vasodilation produced by genistein is antagonized by the NO synthase inhibitor L-NMMA. L-NMMA inhibits both basal release of NO and acetylcholine-stimulated NO release.[11] Inhibition of basal NO could antagonize the actions of genistein if NO potentiated the effects of genistein (or vice versa). However, we did not observe any potentiation of the vasodilator effect of genistein during coinfusion of nitroprusside with a threshold dose of genistein. The simplest and most likely explanation for the effect of L-NMMA on genistein is that the actions of genistein are mediated in part via direct activation of the L-arginine/NO pathway. Other actions, such as relaxation of vascular smooth muscle through inhibition of tyrosine kinase (see later) or nongenomic effects that modulate membrane ion channels and intracellular calcium, as described for estradiol,[12,13] could contribute to the component of the response resistant to L-NMMA.

At physiological concentrations, estrogen acutely improves endothelium-dependent vasodilation in postmenopausal women.[14–16] Estrogen also ameliorates exercise-induced myocardial ischemia in women with coronary artery disease.[17] Although an acute effect has not been seen with similar doses in some studies in men,[18] arterial reactivity is enhanced in men taking high-dose estrogen[19] and conjugated estrogens favorably influence coronary vasomotion in men.[20,21] An acute vasodilator effect of estrogen has not been reported, however, and the second major finding of the present study is that high concentrations of 17β-estradiol produce acute vasodilation in forearm vasculature of healthy men. The actions of estrogen were similar to those of equimolar concentrations of genistein and were antagonized to a similar degree by L-NMMA. In cultured endothelial cells, estrogen activates NOS via a rapid nongenomic action mediated via ER-α.[22] Genistein exhibits lower binding affinity than estrogen to ER-α but similar or greater affinity to ER-β.[23] It is possible, therefore, that the actions of genistein and, in part, those of estrogen are mediated through ER-β via a mechanism similar to that described for estrogen acting through ER-α. We observed genistein to have similar vasodilator effects in men and women. This is consistent with an action through ER-β that is found in high density in vascular tissue from both sexes.[24] The similar responses to estrogen and genistein with regard to the potency of these agonists and the degree to which the response is inhibited by L-NMMA also support an action through ER-β. Few studies to date have addressed which receptor is associated with the cardioprotective actions of estrogen. It is noteworthy, however, that ER-β has also been implicated in the inhibition of the vascular injury response by estrogen in ER-α–deficient mice.[24,25]

A nonspecific action of phytoestrogens in activating NO synthase is excluded by the observation that daidzein (which has low affinity for both ER-α and ER-β) does not produce vasodilation. It is possible, however, that the vasodilator actions of genistein are mediated by a non–estrogen-related mechanism, such as the inhibition of tyrosine protein kinases, although such effects are usually observed at higher concentrations.[26] Definitive in vivo studies to distinguish between such mechanisms and to define the estrogen receptor subtype involved in the vasodilator response to genistein and estrogen in humans require the use of specific antagonists that are not currently available for human use.

Regarding the mechanism by which genistein interacts with the NO pathway, its actions on this pathway could be of functional importance. NO has antiatherogenic actions, including inhibition of monocyte adhesion to the endothelium, inhibition of smooth muscle proliferation, and antiplatelet actions.[27] Inhibition of NO release in animal models results in accelerated atherogenesis.[28,29] Stimulation of NO release by genistein could have cardioprotective actions. Reduction in peripheral vascular resistance by genistein could influence blood pressure and the distribution of blood flow.

We also observed an effect of a threshold dose of genistein in potentiating responses to the endothelium-dependent vasodilator acetylcholine. This potentiation was specific in that responses to the endothelium-independent vasodilator nitroprusside were unaffected by the same dose of genistein. An impaired vasodilator response to acetylcholine is associated with risk factors for CHD and is thought to reflect impaired release or decreased availability of simulated endothelium-derived NO.[30] Estrogen potentiates the acetylcholine vasodilator response in postmenopausal women,[14–16,18] and this action has been suggested to account for cardioprotective effects of estrogen in postmenopausal women (when given unopposed by progestins, which may negate this effect).[31,32] The ability of low-dose genistein to potentiate acetylcholine suggests that genistein may have similar protective actions in facilitating actions of stimulated endothelium-derived NO. Such actions could add to the direct effects of genistein.

The present study supports the suggestion that dietary phytoestrogens that produce high plasma concentrations of genistein contribute to the lower incidence of CHD in Japan compared with in other industrialized nations.[6] The potential feasibility of genistein use to prevent or treat atherosclerosis is highlighted by the finding that plasma concentrations of genistein in subjects who consume a traditional Japanese diet approximate the threshold concentration of genistein that causes NO-dependent vasodilation and potentiates the vaso-

dilator response to acetylcholine in the present study. Some caution must be applied, however, in extrapolation of the results of the present study to infer effects from dietary supplementation. Effects in forearm vasculature may not necessarily be representative of those in other vascular beds. In addition, the concentrations of genistein measured in plasma after dietary supplementation may include conjugates of genistein,[33] and we have not determined whether such conjugates have actions on the vasculature that are similar to those of unconjugated genistein.

In conclusion, we have demonstrated that genistein produces acute NO-dependent vasodilation in the forearm vasculature of men and women with a potency similar to that of 17β-estradiol and potentiates endothelium-dependent vasodilation. Such actions may provide the basis for novel approaches to hypertension and the primary and secondary prevention of atherosclerosis.

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