The Isoflavone Equol Mediates Rapid Vascular Relaxation

*INDEPENDENT ACTIVATION OF ENDOTHELIAL NITRIC-OXIDE SYNTHASE/Hsp90 INVOLVING ERK1/2 AND Akt PHOSPHORYLATION IN HUMAN ENDOTHELIAL CELLS*

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We recently reported that soy isoflavones increase gene expression of endothelial nitric-oxide synthase (eNOS) and antioxidant defense enzymes, resulting in improved endothelial function and lower blood pressure in vivo. In this study, we establish that equol (1–100 nM) causes acute endothelium- and nitric oxide (NO)-dependent relaxation of aortic rings and rapidly (2 min) activates eNOS in human aortic and umbilical vein endothelial cells. Intracellular Ca2+ and cyclic AMP levels were unaffected by treatment (100 nM, 2 min) with equol, daidzein, or genistein. Rapid phosphorylation of ERK1/2, protein kinase B/Akt, and eNOS serine 1177 by equol was paralleled by association of eNOS with heat shock protein 90 (Hsp90) and NO synthesis in human umbilical vein endothelial cells, expressing estrogen receptors (ER)α and ERβ. Inhibition of phosphatidylinositol 3-kinase and ERK1/2 inhibited eNOS activity, whereas pertussis toxin and the ER antagonists ICI 182,750 and tamoxifen had negligible effects. Our findings provide the first evidence that nutritionally relevant plasma concentrations of equol (and other soy protein isoflavones) rapidly stimulate phosphorylation of ERK1/2 and phosphatidylinositol 3-kinase/Akt, leading to the activation of NOS and increased NO production independent of cytosolic Ca2+ levels. Identification of the nongenomic mechanisms by which equol mediates vascular relaxation provides a basis for evaluating potential benefits of equol in the treatment of postmenopausal women and patients at risk of cardiovascular disease.

The interaction of estrogen with estrogen receptors (ER)α, β, and γ leads to the translocation activation of estrogen-responsive genes (1–3), including eNOS (4) and key antioxidant defense genes (5). In addition to its genomic actions, estrogen rapidly stimulates eNOS activity in cultured endothelial cells. ERα, localized in plasmalemmal caveolae, has been implicated in the rapid activation of eNOS via pathways involving ERK1/2 and PI 3-kinase/Akt (6–12). In pulmonary artery endothelial cells, coupling of ERα to Gαi activates downstream signaling pathways leading to NO production (13). Inhibition of the functional association of the chaperone protein Hsp90 with eNOS abolishes 17β-estradiol-stimulated NO release (14), and studies in HUVEC have shown that fluid shear stress stimulates phosphorylation of eNOS via PI 3-kinase/Akt, leading to increased NO production independent of cytosolic Ca2+ mobilization (15). A similar Ca2+ insensitivity has been reported for eNOS activation by 17β-estradiol (E2) in HUVEC (16), although in bovine and human aortic endothelial cells E2 appears to elevate intracellular Ca2+ (17, 18).

Genistein and daidzein are polyphenolic isoflavones contained in soy protein (19), and intestinal bacteria metabolize daidzein to equol. Isoflavones are structurally similar to estrogen and generally bind with higher affinity to estrogen receptor β (ERβ) compared with ERα (20). In humans consuming a soy-deficient diet, plasma concentrations of genistein are <40 nM but can reach 4 μM in Japanese consuming a soy-rich diet (19, 21). Dietary phytoestrogens have been associated with a favorable cardiovascular risk profile in postmenopausal women (22). However, the limited number of clinical trials only describe marginal benefits of isoflavones in healthy postmenopausal women, highlighting the need for further studies with postmenopausal women at risk of diabetes, cardiovascular disease, breast cancer, and osteoporosis (23).

Long term treatment with genistein improves endothelial function in humans and animal models with mild to moderate hypertension (24–27), whereas acute treatment enhances flow-mediated dilation and endothelium-dependent relaxation (28–32). Isoflavones may thus affect vascular tone by regulating

HUVEC, human umbilical vein endothelial cells; ERK, extracellular signal-regulated kinase; PI, phosphatidylinositol; PG, prostaglandin; NOS, nitric-oxide synthase; E2, 17β-estradiol; KPB/S, Kreb’s phosphate-buffered saline; BSA, bovine serum albumin; FCS, fetal calf serum; PE, phenylephrine; IBMX, 3-isobutyl-1-methylxanthine; AMPK, AMP-activated protein kinase; BAE, bovine aortic endothelial cells; PTX, pertussis toxin; l-NAME, Nω-nitro-l-arginine methyl ester.
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either eNOS expression and/or bioavailability of NO (33). We recently reported that a soy protein diet rich in isoflavones increases the expression of eNOS and key antioxidant defense genes in aged male rats, resulting in improved NO-dependent relaxation and reduced arterial blood pressure in vivo (34).

Our present findings provide the first evidence that equol (0.1–100 nM) causes rapid activation of ERK1/2 and NO release in HUVEC and HAEC at basal cytosolic Ca2+ levels and NO-dependent relaxation. Equol-stimulated phosphorylation of Akt and eNOS serine 1177 was rapid (2 min) and accompanied by a dissociation of eNOS from caveolin-1 and association with Hsp90. Rapid activation of eNOS by equol was abrogated following inhibition of ERK1/2 and PI 3-kinase, and was not substantially affected by inhibitors of G protein-coupled receptors, Src family kinases, or the estrogen antagonists ICI 182,780 and tamoxifen.

MATERIALS AND METHODS

Endothelial Cell Culture—Human umbilical vein endothelial cells (HUVEC) were isolated by collagenase digestion (1 mg ml−1) and cultured in low phenol red M199 containing 10% (v/v) fetal calf serum (FCS), 10% (v/v) newborn calf serum, 5 mM l-glutamine, and endothelial cell growth factor (20 μg ml−1) (35, 36). Human aortic endothelial cells (HAEC, Clonetics, Berkshire, UK) were initially maintained in EGM-2 medium (Clonetics) and then passaged in low phenol red M199.

Detection of Estrogen Receptors ERα and ERβ—HUVEC were seeded into chamber slides and cultured overnight in M199. For light microscopy, cells were fixed with 4% paraformaldehyde for 10 min. Fixative was replaced with 0.01 M Kreb’s phosphate-buffered saline (KPBS); one group of cells was permeabilized with KPBS containing 0.1% Triton X-100 for 10 min to facilitate penetration of reagents and another group remained nonpermeabilized. After blocking with 5% bovine serum albumin (BSA), cells were incubated with primary antibodies raised against the carboxyl terminus of human ERα (amino acids 582–595; Calbiochem, 0.5 μg ml−1) or human ERβ (amino acids 458–477; Zymed Laboratories Inc.; D7N, 0.5 μg/ml) for 48 h. Cells were thoroughly rinsed with KPBS and blocked with a mixture of 0.1% cold water fish gelatin (Electron Microscopy Sciences, Hatfield, PA) and 1% BSA in KPBS. Nanogold goat anti-rabbit IgG, Fab′ fragment (Molecular Probes) diluted in the same blocking solution 1:100, was used for 3 h followed by glutaraldehyde fixation (1.25%) in KPBS. Cells were washed in 0.2 M sodium citrate, pH 7.5, and gold particles were silver intensified with IntenSE kit (Amersham Biosciences). Cells were treated with 1% osmium tetroxide in 0.1 M phosphate buffer for 30 min, dehydrated in ascending series of ethanol, embedded in Durcupan ACM epoxy resin (Fluka), and polymerized at 56 °C for 2 days. Ultrathin 50–60 nm thin sections were cut using a Leica ultracut UCT ultramicrotome; ribbons were collected onto Formvar-coated single slot grids and examined with a JEOL electron microscope.

Immunocytochemical controls revealed no punctate staining after processing cells without primary antibodies (Fig. 1, B and D) or when ER antibodies were pre-absorbed with peptide antigens or recombinant proteins.

Single Cell [Ca2+]i Measurements—HUVEC were loaded with 1 μM fura 2-AM for 60 min in HEPES-buffered Dulbecco’s modified Eagle’s medium containing 20% FCS and then maintained in HEPES-buffered balanced salt solution (36, 38). Fura 2-AM-loaded cells were mounted on a thermostated stage (37 °C) of a Zeiss Axiovert 135 inverted microscope with a ×40 oil immersion objective and excited alternately at 350 and 380 nm via a LEP dual filter wheel system (Ludl, Hawthorne, NY). Fluoresced light, >420 nm, was captured with a 14-bit cooled CCD camera (Hamamatsu C4880-80) using OpenLab software (Improvision, Coventry, UK). An image pair was captured every 1–3 s, and the ratio of fluorescence at 350 and 380 nm excitation was used as a measure of intracellular calcium [Ca2+]i.

Relaxation of Isolated Aortic Rings—Thoracic aortae from male adult Wistar rats were rapidly excised, cleaned of connective tissue, and ~2 mm rings mounted onto isometric force transducers in a jacketed (37 °C) organ bath containing Krebs-Henseleit solution (mm): NaCl 118, KCl 6, NaHCO3 25, NaH2PO4 1.2, HEPES 10, CaCl2 1.6, MgSO4 1.2, pH 7.4) gassed with 95% O2 in 5% CO2 (31, 34). Resting tension (1 g) was applied to endothelium intact rings, and endothelial function was confirmed by relaxation of phenylephrine (PE, 1 μM) preconstricted rings to acetylcholine (10−6 M). After equilibration in buffer for 1 h, rings were pretreated with vehicle or the NO inhibitor Nω-nitro-l-arginine methyl ester (l-NAME, 100 μM) and constricted with 1 μM PE, and relaxation to increasing doses of equol (0.01–10 μM) was monitored.

Intracellular cGMP Accumulation—Basal and stimulated cGMP levels were determined by radioimmunoassay, with inhibition of cGMP accumulation by l-NAME (100 μM, 15 min) serving as an index of NO production (36). Confluent monolayers were washed twice with warmed Krebs-Henseleit buffer and then preincubated for 15 min with buffer containing l-arginine (100 μM) and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 500 μM). Cells were then stimulated (30 s to 15 min) with 1–100 nM equol, daidzein, or genistein in the presence of IBMX and l-arginine (100 μM), and responses were compared with E2 or E2 conjugated to bovine albumin (E2-BSA).
In other experiments, cells were pretreated with inhibitors of PI 3-kinase ([2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride], LY294,002, 10 μM, 30 min), Src kinase (4-amino-5-(4-chlorophenyl)-7(t-buty1)pyrazolo-[3,4-d]pyrimidine (PP2, 10 μM, 30 min), pertussis toxin (100 ng ml⁻¹, 120 min), or the estrogen receptor antagonists ICI 182,780 (10 μM, 30 min) and tamoxifen (10 μM, 60 min). Pretreated cells were then stimulated acutely with isoflavones or E2 in the presence of L-arginine (100 μM) and IBMX (0.5 mM) and vehicle or inhibitor(s). Prostacyclin (PGL₂) release into the supernatant was analyzed by radioimmunoassay of 6-keto-PGF₁α, the stable metabolite of PGI₂ (36).

Immunoblotting Phosphorylated ERK1/2, Akt, and eNOS Ser1177—HUVEC were equilibrated in M199 containing 1% FCS for 4 h and then preincubated with Krebs-Henseleit buffer containing L-arginine (100 μM) and IBMX (0.5 mM). In other experiments, HUVEC were pretreated with vehicle or U0126 (1 μM, 30 min) or LY294,002 (10 μM, 30 min) and then stimulated for 30 s to 5 min with equol, using thrombin (1 unit/ml, 2 min) as an internal control. We reported previously (36) that the structurally distinct MEK1/2 inhibitors U0126 and PD98059 do not have inhibitory effects on p38MAPK or c-Jun NH2-terminal kinase (JNK) pathways in HUVEC. Reactions were stopped with ice-cold PBS containing 200 mM sodium orthovanadate, and cell lysates were separated by SDS-PAGE, and proteins were electrotransferred onto polyvinyldifluoride membranes and then probed with a polyclonal antibody against dually phosphorylated (threonine 183/tyrosine 185) ERK1/2 or phosphospecific antibodies against Akt or eNOS serine 1177. Protein bands were detected by ECL, and densitometric analyses were performed using Scion Image software (Scion Corp.).

Immunoprecipitation of eNOS, Association with Hsp90, and Dissociation from Caveolin-1—HUVEC in T75 culture flasks were equilibrated in M199 containing 1% FCS for 4 h prior to treatment for 2 min with equol (100 nm) or thrombin (1 unit ml⁻¹) in Krebs-Henseleit buffer containing

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L-arginine (100 μM) and IBMX (0.5 mM). Cells were washed with ice-cold PBS, and cell lysates were collected in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing a protease inhibitor mixture (Sigma). Lysates were centrifuged at 13,000 × g for 10 min, and supernatants were incubated overnight at 4 °C with ExactaCruz immunoprecipitation matrix (Santa Cruz Biotechnology) precoated with an eNOS antibody. Immobilized immune complexes were washed twice with RIPA buffer, and then analyzed by SDS-PAGE using antibodies against eNOS, caveolin-1, and Hsp90.

Cell Culture Reagents and Materials—All cell culture reagents, collagenase type II from Clostridium histolyticum, E2, E2-BSA, histamine, thrombin, IBMX, rolipram, L-NAME, ICI 182,780, tamoxifen, and pertussis toxin were obtained from Sigma; equol was from Apin Chemicals Ltd. (UK); daidzein and genistein were from Alexis Biochemicals; fura-2 acetoxymethyl ester (fura2-AM) was from Molecular Probes (Cambridge Bioscience, UK); U0126, LY294002, and antibodies against ERK1/2 were from Promega (Southampton, UK); antibodies against eNOS, Hsp90, and caveolin-1 were from Santa Cruz Biotechnology, and α-tubulin was from Chemicon. ECL reagents, cyclic AMP, 125I-labeled 3',5'-cyclic GMP, and prostacyclin EIA kits were from Amersham Biosciences.

Statistical Analysis—Data are expressed as means ± S.E. of measurements in 3–7 different endothelial cell cultures. Statistical analysis was performed by use of analysis of variance followed by a Student’s t test, and a level of p < 0.05 was considered significant.

RESULTS

Intracellular Localization of ERα and ERβ in HUVEC—Mild fixation and permeabilization with Triton X-100 were used to detect ERα or ERβ immunoreactivity within the nucleus of HUVEC (Fig. 1, A and C), with staining absent when the primary antibody was omitted (Fig. 1, B and D). Stronger fixation of cells not treated with Triton X-100 showed lower signal intensity within the nucleus, but immunoreactive loci were preserved throughout the cytoplasm (Fig. 1, E and F). Doubly labeled immunofluorescence showed ERα and ERβ reactivity in different loci of the cell (Fig. 1, G and H), and electron microscopy revealed ERβ reactivity in the nucleus and cytoplasm of cells, with a few immunoreactive loci also detected at the plasma membrane (Fig. 1, I and J).

Acute Effects of Equol and Histamine on Mobilization of [Ca2+]i—To examine whether equol acutely alters [Ca2+]i in human endothelial cells, fura 2-AM (1 μM)-loaded HUVEC were challenged for 2 min with equol and then histamine. As shown in Fig. 1K, equol (10 or 100 nM) failed to elevate [Ca2+]i, whereas histamine (100 μM) evoked a classical biphasic increase in [Ca2+]i (36, 38). Closer examination of single cells revealed no localized [Ca2+]i responses to equol. Similar responses were observed following acute (2 min) application of 17β-estradiol (data not shown), confirming previous findings in HUVEC (16).

Equol Causes Rapid Endothelium- and NO-dependent Relaxation—Endothelium intact aortic rings were preconstricted with PE (1 μM) and exposed to cumulative doses of equol. As shown in representative original records (Fig. 2, A and B), equol caused a dose-dependent relaxation that was inhibited by pretreatment with L-NAME (100 μM). Fig. 2C summarizes the results from three different animals.

Isoflavones Do Not Acutely Increase cAMP or PGI2 Release—Treatment of HAEC (data not shown) or HUVEC for 2 min with 100 nM equol, daidzein, or genistein had no significant effect on intracellular cAMP levels or PGI2 production, whereas forskolin and histamine increased cAMP levels and PGI2 production, respectively (Table 1).

Rapid Activation of ERK1/2 and eNOS by Equol, Daidzein, and Genistein—Because ERK1/2 has been implicated in the activation of eNOS by estrogen (6, 10, 12), we compared acute effects of E2, equol, daidzein, and genistein on ERK1/2 phosphorylation in HUVEC and HAEC. Treatment with isoflavones (100 nM) led to a rapid (2 min) phosphorylation of ERK1/2 (Figs. 3, A and C) in both cell types, and densitometric analyses of different cell cultures are shown in Fig. 3, B and D.

We reported previously that activation of A2a-purinoceptors results in rapid phosphorylation of ERK1/2 and NO production in HUVEC independent of detectable increases in cytosolic...
Ca²⁺ (36). To examine whether equol, daidzein, or genistein also acutely stimulate NO synthesis in endothelial cells, we treated HUVEC and HAEC with these isoflavones (100 nM, 2 min) and measured changes in intracellular cGMP accumulation in the absence or presence of L-NAME (100 μM). Equol, daidzein, and genistein (and E₂) evoked rapid increases in cGMP accumulation, which were inhibited by L-NAME (Fig. 3E).

We then further examined concentration- and time-dependent actions of equol on ERK1/2 phosphorylation and cGMP accumulation in HUVEC. ERK1/2 was phosphorylated by equol concentrations as low as 1 nM (data not shown), with phosphorylation at higher concentrations (100 nM) detected within 30 s and maintained for up to 5 min (Fig. 4, A and B). Activation of ERK1/2 was paralleled by an increase in intracellular cGMP, with maximal NO release achieved at 2 min (Fig. 4C). All subsequent experiments were performed using 100 nM isoflavones or E₂.

**ERK1/2 Inhibition Abrogates Equol-mediated NO Synthesis**

Preincubation of HUVEC with the MEK1/2 inhibitor U0126 (1 μM, 30 min) abolished ERK1/2 phosphorylation in response to acute treatment with 100 nM equol, daidzein, genistein, or E₂ (Fig. 5A), with similar findings observed in HAEC (data not shown). Under the same experimental conditions, no detectable ERK1/2 phosphorylation was observed in the presence of L-NAME (Fig. 5B). These findings suggest that ERK1/2 activation is a critical upstream mediator of equol-mediated NO synthesis.

**TABLE 1**

| Treatment       | cAMP   | PGI₂   |
|-----------------|--------|--------|
|                 | fmol/well | pg/well |
| Control         | 29 ± 13 | 42 ± 13 |
| Equol (100 nM)  | 37 ± 15 | 45 ± 4  |
| Daidzein (100 nM)| 25 ± 11 | 51 ± 13 |
| Genistein (100 nM)| 33 ± 19 | 30 ± 12 |
| Forskolin (1 μM) | 140 ± 36*** | ND      |
| Histamine (10 μM) | ND      | 140 ± 29** |

Preincubation of HUVEC with the MEK1/2 inhibitor U0126 (1 μM, 30 min) abolished ERK1/2 phosphorylation in response to acute treatment with 100 nM equol, daidzein, genistein, or E₂ (Fig. 5A), with similar findings observed in HAEC (data not shown). Under the same experimental conditions, no detectable ERK1/2 phosphorylation was observed in the presence of L-NAME (Fig. 5B). These findings suggest that ERK1/2 activation is a critical upstream mediator of equol-mediated NO synthesis.

**FIGURE 3. Isoflavones acutely activate ERK1/2 and eNOS in HUVEC and HAEC.** Serum-deprived endothelial cells were incubated in Krebs-Henseleit buffer containing l-arginine (100 μM) and then stimulated for 2 min with vehicle control (Ctrl) (0.01% Me₂SO) or 100 nM equol (Eq), daidzein (Daid), genistein (Gen) or E₂, using thrombin (Thr) (1 unit ml⁻¹) as a positive control. Representative immunoblots of dually phosphorylated ERK1/2 relative to α-tubulin in HUVEC (A) and HAEC (C) cell lysates with densitometric analyses of three different HUVEC (B) and HAEC (D) cultures shown. All values for stimulated ERK1/2 phosphorylation in B and D are significantly different from control (means ± S.E., n = 4, p < 0.05). E, basal and stimulated cGMP levels in HUVEC monolayers pretreated for 15 min with vehicle or l-NAME (100 μM) and then stimulated for 2 min with 100 nM equol, daidzein, genistein, or E₂ in the continued absence (filled bars) or presence (unfilled bars) of l-NAME (100 μM). cGMP accumulation is expressed as means ± S.E. or replicate measurements in each of 3–7 different cell cultures. *, p < 0.05 versus control; †, p < 0.05 versus respective treatments in absence of l-NAME.
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**Figure 4.** **Equol stimulates acute ERK1/2 phosphorylation and NO production in HUVEC.** A, serum-deprived cells were stimulated for 2 min with vehicle or equol (0.1 and 100 nM), and lysates were immunoblotted for phosphorylated ERK1/2 relative to α-tubulin. A, time course of ERK1/2 activation by 100 nM equol with densitometric analysis of three different cell cultures as shown in B, C, time course of cGMP accumulation induced by 100 nM equol. Data are expressed as means ± S.E. of measurements in each of four (ERK1/2) or seven (cGMP) different cell cultures. Stipulated cGMP levels (1–5 min) are significantly different versus control (Ctrl) at t = 0 min, p < 0.05.

**Figure 5.** Effects of MEK1/2 inhibition on acute activation of ERK1/2 and eNOS by equol and other isoflavones. Serum-deprived HUVEC were pretreated with U0126 (1 μM, 30 min) and then stimulated for 2 min with vehicle or 100 nM equol (Eq), daidzein (Daid), genistein (Gen), or E2 in the absence or presence of U0126 (1 μM). A, cell lysates were immunoblotted for dually phosphorylated ERK1/2 and α-tubulin. B, HUVEC were treated for 2 min with 100 nM isoflavones or E2 in the absence (filled bars) or presence (unfilled bars) of U0126 (1 μM, 30 min), and changes in cGMP accumulation are expressed as percentage change over control (basal cGMP levels ranged from 0.6 to 12.3 pmol (10⁶ cells⁻¹)). Data are expressed as means ± S.E. of duplicate measurements in each of four different cell cultures. *, p < 0.01 versus control (Ctrl); #, p < 0.05 versus stimulation.

**Effect of Pertussis Toxin on Isoflavone-stimulated ERK1/2 Phosphorylation and cGMP Accumulation**—Previous studies have shown that activation of eNOS by E2 requires plasma membrane ERα coupling to Gα, leading to activation of downstream signaling events (13). To evaluate the potential role of G proteins in equol-stimulated eNOS activity, HUVEC were pretreated for 120 min with vehicle or pertussis toxin (PTX) and then stimulated for 2 min with equol (100 nM) or thrombin (1 unit ml⁻¹) resulted in a rapid dissociation of eNOS from caveolin-1 and association with Hsp90 (Fig. 6H).

**Effect of Src Inhibition on ERK1/2 and eNOS Activation**—Src kinase has been implicated in the activation of Akt and eNOS in...
HUVEC in response to E2 (41). To examine the potential involvement of Src kinase in the actions of equol and other isoflavones, HUVEC were pretreated for 30 min with an Src kinase inhibitor PP2 (10 μM). PP2 abrogated acute phosphorylation of ERK1/2 in response to equol, daidzein, and genistein (100 nM, 2 min) but had negligible effects on stimulated NO release (data not shown).

**Estrogen Receptor Antagonists Do Not Inhibit Acute Activation of ERK1/2 and eNOS by Equol and Other Isoflavones**—In view of previous reports for E2 (11), we hypothesized that estrogen receptor antagonists would inhibit isoflavone-stimulated activation of ERK1/2 and eNOS. Pretreatment of HUVEC with ICI 182,780 (10 μM) or tamoxifen (10 μM) had no effect on acute (2 min) phosphorylation of ERK1/2 or cGMP accumulation in response to 100 nM equol, daidzein, genistein, or E2 (Fig. 8, A and B).

To further evaluate whether estrogen receptor antagonists affected equol and E2-stimulated cGMP accumulation over a longer time interval, we pretreated HUVEC with ICI 182,780 (10 μM) and then measured intracellular cGMP after 15 min of treatment with either equol or E2. As shown in Fig. 8C, ICI 182,780 inhibited E2 but not equol-stimulated cGMP production, confirming earlier reports of inhibition of E2-stimulated eNOS activity (after 15–30 min) in human endothelial cells by ER antagonists (9, 11, 14).

**DISCUSSION**

We have demonstrated that nutritionally relevant plasma concentrations of equol cause rapid activation of ERK1/2 and Akt in human endothelial cells, leading to phosphorylation of eNOS, NO release, and endothelium-dependent relaxation of aortic rings. Although HUVEC express both ERα and ERβ, acute stimulation of NO synthesis was insensitive to inhibition by the ER antagonists ICI 182,780 and tamoxifen. Rapid activation of eNOS in HUVEC by equol at basal cytosolic Ca2+ levels is consistent with previous reports of shear stress and E2-stimulated NO production at low intracellular Ca2+ (15, 16). Moreover, as reported for E2 (14, 39, 40), equol causes a rapid dissociation of eNOS from caveolin-1 and association with the chaperone protein Hsp90. To our knowledge, our findings provide the first evidence that equol, a metabolite of the isoflavone daidzein, enhances association of Hsp90 with eNOS in human endothelium.

We have shown, for the first time, a similar distribution of ERα and ERβ immunoreactivity in HUVEC, confirming earlier reports of immunocytochemical staining (42) and detection of mRNA and protein for ERα in HUVEC (14). After mild fixation, immunoreactivity for both receptors appears in the nuclei of HUVEC, implicating a genomic function. Fixation of cells without permeabilization revealed punctate immunoreactivity over both extranuclear and nuclear sites. Such strategy has been used to detect estrogen receptors at extranuclear sites in other cell types (43). Because ERα and ERβ can form heterodimers (44–46), we further examined whether receptors were co-localized at immunoreactive sites. Negligible co-localization at nuclear or extranuclear sites suggests that formation of ER complexes may not required for acute activation of eNOS in HUVEC in response to equol or E2. A study of en face arterial endothelium has also revealed negligible co-localization of ERα...
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FIGURE 7. Effects of pertussis toxin on isoflavone-stimulated activation of ERK1/2 and eNOS in HUVEC. Serum-deprived cells were pretreated with PTX (100 ng/ml), 120 min) and then stimulated for 2 min with vehicle or 100 nM equol or E2-conjugated to bovine serum albumin (E2-BSA). A, immunoblot for phosphorylated ERK1/2 relative to α-tubulin is representative of two different cell cultures. B, PTX (100 ng/ml, 120 min) pretreatment attenuates, but does not abolish, isoflavone- or E2-BSA (100 nM)-stimulated cGMP accumulation. Data are expressed as means ± S.E. of replicate measurements in each of four different cell cultures. All stimulated cGMP levels were significantly different from control (*, p < 0.05; †, p < 0.01 versus stimulation with Daid in absence of PTX).

FIGURE 8. Acute stimulation of NO production in HUVEC by equol and other isoflavones is unaffected by estrogen receptor antagonists. Cells were pretreated with the estrogen receptor antagonists ICI 182,780 (10 μM, 30 min) or tamoxifen (10 μM, 30 min), incubated in Krebs-Henseleit buffer containing L-arginine (100 μM) and IBMX (0.5 mM), and then stimulated for 2 min with 100 nM equol (Eq), daidzein (Daid), genistein (Gen), or E2 in the continued presence of the estrogen receptor antagonists. A, cell lysates were immunoblotted for dually phosphorylated ERK1/2 (not shown), and bar graph summarizes densitometric analysis of data from three different cell cultures. B, pretreatment of cells with ICI 182,780 (10 μM) or tamoxifen (10 μM) does not inhibit acute stimulation of cGMP accumulation by Eq, Daid, or E2 (100 nM, 2 min). Control basal cGMP values ranged from 2 to 6.6 pmol (10^6 cells)^{-1}. C, Effects of prolonged treatment with equol or E2 (100 nM, 15 min) on cGMP accumulation in cells pretreated with vehicle or ICI 182,780 (10 μM). Changes in cGMP accumulation are expressed as a percentage of control and denote means ± S.E. of duplicate measurements in each of four different cell cultures. All stimulated values in A and B were significantly different from control (Ctrl) (p < 0.05). C, *, †, ‡, p < 0.05 versus control (Ctrl). Control cGMP values ranged from 2 to 6.6 pmol (10^6 cells)^{-1}.

We have shown previously in HUVEC that vasoactive agonists such as histamine activate eNOS via an increase in cytosolic Ca^{2+} (38), whereas A2A-purinoceptor stimulation elicits rapid Ca^{2+}-independent and ERK1/2-dependent NO release (36). We report here that acute treatment of HUVEC with equol does not alter cytosolic Ca^{2+} (Fig. 1K), reminiscent of E2-mediated activation of eNOS independent of Ca^{2+} mobilization (16). However, acute regulation of NO synthesis varies in endothelial cells from different vascular beds or species, because E2 causes a transient rise in cytosolic Ca^{2+} in bovine aortic and human arterial endothelial cells (17, 18). Ca^{2+}-independent activation of eNOS in HUVEC in response to equol (Fig. 1K), E2 (14, 16), adenosine (36), or shear stress (15) may reflect the ability of phosphorylation to activate eNOS at resting cytosolic Ca^{2+} levels by enhancing electron flux through the reductase domain of the enzyme and reducing calmodulin dissociation (51).

We have also demonstrated that nanomolar concentrations of equol, daidzein, and genistein acutely (30 s to 2 min) stimulate phosphorylation of ERK1/2 and NO synthesis in HUVEC and HAEC. Increases in NO synthesis were inhibited by the NOS inhibitor l-NAME. As eNOS inhibition had no effect on equol-stimulated ERK1/2 phosphorylation (data not shown), this suggests that activation of ERK1/2 lies upstream of eNOS. Inhibition of ERK1/2 with U0126 significantly attenuated equol-stimulated eNOS serine 1177 phosphorylation and NO production. Previous studies established that E2 causes a rapid-
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Isolavones may act as inhibitors of cAMP phosphodiesterase (55). However, as cAMP levels in HUVEC were unaffected by acute treatment with equol and other isolavones, effects of cAMP-dependent protein kinase inhibitors on eNOS activation by isolavones were not examined. Previous studies with bovine aortic endothelial cells (BAEC) reported that longer term treatment with genistein (100 nM, 10–120 min) stimulates activation of eNOS independent of ERK1/2, PI 3-kinase/Akt, or the involvement of estrogen receptors (56). The lack of inhibition of genistein-stimulated eNOS activity by ICI 182,780 is consistent with our present results. The discrepancies between our findings and those of Liu et al. (56), based primarily on experiments with BAEC, may reflect differences in cell type, speciess, and/or the time interval of genistein treatment (30 s versus 10 min). Moreover, Liu et al. (56) only reported increases in cAMP levels in BAEC in response to 10 nM to 100 μM genistein. In our study, treatment of HUVEC with isolavone concentrations of >100 nM actually inhibited ERK1/2 phosphorylation and did not lead to enhanced NO synthesis (data not shown). The inhibition of tyrosine kinase activity by genistein (10−50 μM) is well known (57), and our present and previous results in HUVEC (36) highlight the concentration-dependent actions of genistein and other isolavones on ERK1/2 activity.

Natural polyphenolic compounds in black and green tea and red wine also activate eNOS (58–60). Epigallocatechin-3-gallate (40−100 μM) stimulates NO release in bovine endothelial cells only after 15 min, with moderate activation of Akt and eNOS serine 1177 (58). Epigallocatechin-3-gallate-stimulated phosphorylation of eNOS was abolished by Akt and cAMP-dependent protein kinase inhibitors but notably unaffected by ERK1/2 inhibition. In porcine endothelial cells, black tea polyphenols stimulate Akt and eNOS phosphorylation within 5 min, involving a Ca2+- and p38MAPK-dependent activation of PI 3-kinase/Akt (59). Red wine polyphenols rapidly activate ERK1/2 and NO production in several endothelial cell types (60) and stimulate PI 3-kinase/Akt-dependent phosphorylation of eNOS in porcine coronary arteries (61). Although actions of trans-resveratrol on ERK1/2 and NO production in cultured endothelial cells are inhibited by ICI 182,780 and tamoxifen (60), studies with tea polyphenols have not assessed the potential inhibitory actions of ER antagonists.

Although pertussis toxin inhibited isoflavone-stimulated ERK1/2 phosphorylation, cGMP levels were only reduced marginally, suggesting activation of eNOS via PI 3-kinase/Akt. The lack of significant inhibition of equol-stimulated NO production by pertussis toxin contrasts with previous studies with E2 in ovine endothelial cells, and suggests that rapid stimulation of

phosphorylation of ERK1/2 and eNOS (6–12), but to our knowledge this is the first report that the isoflavones equol, daidzein, and genistein stimulate ERK1/2 and eNOS phosphorylation in human endothelium within 2 min.

Activation of the serine/threonine kinase Akt, a downstream target of PI 3-kinase, leads to rapid phosphorylation of eNOS and NO release (6–12, 16, 52, 53). When we examined the effect of a PI 3-kinase inhibitor in HUVEC, we found that LY294,002 markedly inhibited equol-stimulated Akt (Fig. 6E) and eNOS serine 1177 phosphorylation. Pretreatment of HUVEC with inhibitors of both PI 3-kinase and ERK1/2 completely abrogated eNOS phosphorylation (Fig. 6G), highlighting the importance of PI 3-kinase/Akt signaling in eNOS phosphorylation (9, 11, 52, 53). E2 rapidly induces Src kinase activity at basal cytosolic Ca2+ levels via ERK1/2 and PI 3-kinase/Akt-dependent pathways. Equol-stimulated release of NO is paralleled by a rapid dissociation of eNOS from caveolin-1 and association with the scaffolding protein Hsp90. Although ERα and ERβ are expressed in HUVEC, the rapid actions (≤2 min) of equol, daidzein, and genistein appear not to be mediated via the coupling of ER receptors to G protein Hsp90. Although ERα/ERβ complexation of the enzyme and eNOS-dependent O2− generation by E2 appears to mediate eNOS activation.

![FIGURE 9. Signaling pathways involved in the rapid activation of eNOS and NO production by equol, daidzein, and genistein in human endothelial cells.](Image)
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eNOS by isoflavones in HUVEC is not mediated via G protein-coupled receptors.

We found that isoflavone-stimulated eNOS activation was independent of ERs, because the classical ER antagonists ICI 182,780 and tamoxifen failed to inhibit equol-, daidzein-, and genistein-stimulated cGMP production. The concentration and preincubation times used in our study are similar to those in previous studies examining the actions of E2. The only difference is the much shorter treatment (2 min versus 10–30 min) used in our experiments. When we compared the inhibitory action of ICI 182,780 on E2- and equol-stimulated cGMP accumulation over 15 min, the ER antagonist selectively inhibited the actions of E2, confirming that equol-stimulated NO production was independent of ER activation. As longer term responses to 17β-estradiol were inhibited by ICI 182,750, this implicates interactions of 17β-estradiol with the classical ERs detected in HUVEC in this study (Fig. 1).

In summary, this study in human umbilical vein endothelial cells establishes that rapid activation of ERK1/2 and PI 3-kinase/Akt signaling pathways by equol, daidzein, and genistein is paralleled by phosphorylation of eNOS serine 1177, Hsp90 association with eNOS, and NO release (Fig. 9). Furthermore, rapid phosphorylation of eNOS serine 1177 in response to equol is abrogated by inhibitors of ERK1/2 and PI 3-kinase, and we have demonstrated cross-talk between these two signaling pathways (Fig 6, E and F). Based on our present findings, we cannot exclude the possibility that equol also modulates NO synthesis via other NOS isoforms expressed at much lower levels in subcultured HUVEC (62, 63). Activation of tyrosine kinases and Hsp90 may act in concert to regulate the balance of NO and O2− generation by eNOS (54). In this context, prolonged treatment of J774 cells with equol increases the bioavailability of NO by inhibiting O2− production (64). We have also documented that equol induces endothelium- and NO-dependent relaxation of isolated aortic rings. Similar studies have reported that dilatory responses to dehydroequol in isolated rat aortic rings are insensitive to inhibition of eNOS (65), whereas dilatation of human forearm resistance arteries is largely NO-dependent (66).

Equol is derived from the soy isoflavone daidzein, and humans have acquired the ability to exclusively synthesize (S)-equol (67). Equol and other isoflavones may activate similar intracellular signaling pathways as a result of their phenolic structure. In a recent study assessing bioavailability and metabolism of soy isoflavones, it was noted that only 30% of the subjects were equol producers with negligible differences in equol production observed with age or gender (68). Thus, use of dietary sources of isoflavones as alternative treatment for postmenopausal women and patients at risk of cardiovascular disease will require evaluation of the ability of subjects to metabolize daidzein to equol. Our findings provide insight into the signaling pathways regulating eNOS activity in human endothelium and identify equol as a potent activator of acute NO production.

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