Equol-Stimulated Mitochondrial Reactive Oxygen Species Activate Endothelial Nitric Oxide Synthase and Redox Signaling in Endothelial Cells
Roles for F-Actin and GPR30

David J. Rowlands, Sarah Chapple, Richard C.M. Siow, Giovanni E. Mann

Abstract—We reported previously that dietary isoflavones modulate arterial blood pressure in vivo and that the daidzein metabolite equol rapidly activates endothelial NO synthase (eNOS) via Akt and extracellular signal–regulated kinase 1/2–dependent signaling. In this study, we report the first evidence in human endothelial cells that acute stimulation of mitochondrial superoxide generation by equol (100 nmol/L) is required for eNOS activation. Scavengers of superoxide (superoxide dismutase and manganese [III] tetrakis[1-methyl-4-pyridyl]porphyrin) abrogated equol stimulated Akt and eNOS phosphorylation, and the mitochondrial complex I inhibitor rotenone inhibited Akt, extracellular signal–regulated kinase 1/2, and eNOS phosphorylation, as well as NO-mediated increases in intracellular cGMP. Equol also induced rapid alterations in F-actin fiber distribution, with depolymerization of F-actin with cytochalasin D abrogating equol-stimulated mitochondrial superoxide generation. Treatment of cells with pertussis toxin or inhibition of GPR30/epidermal growth factor receptor kinase transactivation prevented equol-induced activation of extracellular signal–regulated kinase 1/2 via c-Src, Akt, and eNOS. Moreover, inhibition of epidermal growth factor receptor kinase activation with AG-1478 abrogated equol-stimulated mitochondrial reactive oxygen species generation and subsequent kinase and eNOS activation. Our findings suggest that equol-stimulated mitochondrial reactive oxygen species modulate endothelial redox signaling and NO release involving transactivation of epidermal growth factor receptor kinase and reorganization of the F-actin cytoskeleton. Identification of these novel actions of equol may provide valuable insights for therapeutic strategies to restore endothelial function in cardiovascular disease. (Hypertension. 2011;57:833-840.) ● Online Data Supplement

Key Words: equol ■ isoflavones ■ endothelium ■ eNOS ■ mitochondria ■ cytoskeleton ■ redox signaling

Dietary soy products contain significant amounts of genistein and daidzein,1,2 which are structurally similar to estrogen, with a preferential affinity for estrogen receptor (ER)-β.3 Plasma concentrations of genistein and other isoflavones are as low as 40 nmol/L in humans consuming low-isoflavone diets but can reach ∼4 μmol/L in Japanese consuming a traditional soybean-rich diet.4,5 The isoflavone equol is produced by metabolism of daidzein by intestinal gut microbiota in only 40% to 50% of individuals,6 but equol is more bioavailable than either genistein or daidzein and reaches plasma concentrations of ∼100 nmol/L in vivo.5 Cardiovascular benefits of supplementation with genistein and tetrahydrodaidzein have been observed,7,8 and we reported previously that dietary isoflavones (genistein, daidzein, and equol) can modulate blood pressure in vivo, antioxidant and endothelial NO synthase (eNOS) gene expression, and intracellular glutathione levels in male rats.9 We further demonstrated that feeding aging male rats an isoflavone-enriched diet improves agonist-mediated endothelium-derived hyperpolarizing factor production in small resistance arteries.10 A recent study in healthy postmenopausal women established that an isoflavone-enriched, low-fat meal increases endothelium-dependent relaxation in vivo within 5 to 7 hours.11 Notably, genistein and dehydroequol elicit rapid endothelium-dependent increases in forearm blood flow in vivo,12,13 and equol relaxes preconstricted rat aortic rings.14 In human fetal endothelial cells, equol acutely stimulates endothelial NO release at basal cytosolic Ca2+ levels via activation of extracellular signal–regulated kinase (ERK) 1/2 and protein kinase B (Akt) but independent of classic ER signaling.14

ERs, aside from their classic function as transcription factors, mediate rapid activation of second messenger systems and intracellular kinases.15 Recently, nonclassic membrane-localized ERs have been identified and appear to...
mediate the rapid actions of 17β-estradiol on intracellular signaling cascades.16,17 The orphan G protein–coupled receptor (GPR30) is a 7-transmembrane spanning G protein–coupled receptor18,19 that binds both 17β-estradiol and genistein.20 GPR30 has been studied primarily in cancerous cells21; however, vascular cells also express the receptor.22 Activation of GPR30 induces transactivation of the epidermal growth factor receptor (EGFR) via Gβγ. Once activated, EGFR stimulates ERK1/2 via activation of c-Src and the phosphoinositide 3-kinase (PI3K)/Akt pathway.23,24

In addition to GPR30 activation, emerging evidence implicates reactive oxygen species (ROS) as second messengers in the activation of PI3K, ERK1/2, c-Src, and EGFR tyrosine kinase.25–27 Although the mitochondrial respiratory chain represents a major source of ROS in the endothelium,28 to our knowledge there are no reports linking activation of eNOS by equol with increased mitochondrial ROS generation. We hypothesized that modulation of GPR30/EGFR, F-actin cytoskeleton, and mitochondrial ROS generation by equol may account for the acute activation of eNOS.

We report the first evidence that inhibition of mitochondrial ROS abolishes equol-induced activation of Akt, ERK1/2, eNOS phosphorylation, and NO production. Our findings link equol-stimulated mitochondrial ROS generation with EGFR transactivation, because inhibition of EGFR activity inhibits kinase activation and eNOS phosphorylation. Furthermore, depolymerization of the F-actin cytoskeleton, known to interact with both EGFR and mitochondria, abrogates mitochondrial ROS generation. Our study provides a novel link between equol-mediated EGFR activation and downstream signal transduction involving mitochondrial ROS in the activation of eNOS.

Methods

For a more detailed Methods description for immunoblotting, quantitative RT-PCR, and cGMP ELSIA, as well as chemicals and reagents, please see the online Data Supplement at http://hyper.ahajournals.org.

Endothelial Cell Culture

Human umbilical vein endothelial cells (HUVECs) were isolated by collagenase digestion (1 mg/mL) and cultured in low phenol red M199 containing 10% (v/v) FCS, 10% FCS (v/v) newborn calf serum, and 5 mmol/L of l-glutamine and endothelial cell growth factor (20 μg/mL).14

Chemiluminescence Detection of ROS Generation in Intact Endothelial Cells

Confluent HUVEC monolayers were incubated in low serum (1% FCS) M199 for 4 hours and then preincubated for 30 minutes in Krebs buffer (in mM: NaCl 118.0, KCl 6.0, NaHCO3 25.0, NaH2PO4 1.2, HEPES 5.0, d-glucose 10.0, CaCl2 1.6, and MgSO4 1.2 [pH 7.4]) containing l-arginine (100 μmol/L) in the absence or presence of superoxide (O2−) dismutase (SOD; 200 U/mL), polyethylene glycol-SOD (PSOD; 50 U/mL), polyethylene glycol-catalase (PCAT; 200 U/mL), manganese (III) tetrakis(1-methyl-4-pyridyl)porphyrin (Mn(100 μmol/L), or rotenone (2 μmol/L). Cells were then incubated in Krebs buffer containing lucigenin (5 μmol/L) and NADPH (100 μmol/L) and challenged with equol (100 nmol/L) or vehicle (0.01% dimethyl sulfoxide [DMSO]) in the absence or presence of inhibitors. Luminescence was immediately recorded in a microplate luminometer (Chameleon V, Hidex) at 37°C after the addition of lucigenin.29 Maximal luminescence values obtained over a 20- to 40-minute interval (see Figure 1A) were averaged for each treatment condition, and values from 3 to 4 independent cell cultures were expressed as mean light units per milligram of protein.

Mitochondrial ROS Production Measured Using MitoSOX Red Fluorescence

Mitochondrial ROS production was measured using the fluorogenic dye MitoSOX Red, a mitochondrially targeted derivative of hydroethidine.30 HUVECs on glass cover slips were loaded with MitoSOX Red (5 μmol/L) for 30 minutes. Cells were subsequently treated in duplicate for 20 minutes with equol (100 nmol/L) or vehicle (0.01% DMSO), and fluorescence was detected in 4% paraformaldehyde-fixed cells by confocal microscopy (560/625 nm). Fluorescence images were obtained from a total of 200 cells per cover slip in each of 4 cultures from 4 different donors. In other experiments, cells were pretreated with the cytoskeletal disrupting agent cytochalasin D (2.5 μmol/L) or EGFR tyrosine kinase inhibitor AG-1478 (5 μmol/L) and then stimulated acutely with equol (100 nmol/L) and monitored with MitoSOX Red fluorescence.

F-Actin Staining With Rhodamine-Phalloidin

Alterations in F-actin cytoskeletal distribution were visualized in fixed cells stained with rhodamine-phalloidin, as described previously.31 Cells were treated with control, vehicle (0.01% DMSO), or equol (100 nmol/L) for 20 minutes, fixed, polymerized F-actin fibers stained with rhodamine-phalloidin (500 nmol/L) for 2 hours at room temperature, and nuclei counterstained with Hoechst (10 μg/mL) for 1 minute. Fluorescence was detected by confocal microscopy with sequential acquisition at wavelengths of 560/625 nm and 375/450 nm used to visualize F-actin and nuclei staining, respectively. In other experiments, cells were pretreated for 30 minutes with cytochalasin D (2.5 μmol/L) before cotreating cells for 20 minutes with equol (100 nmol/L) in the continued absence or presence of cytochalasin-D (2.5 μmol/L).

Statistical Analysis

Data are expressed as mean±SEM of measurements in 3 to 5 different HUVEC cultures obtained from different donors, unless stated otherwise. Statistical analyses were performed using a Student 2-tailed t test or 1-way ANOVA followed by Dunnett multiple comparison, with P<0.05 considered statistically significant.

Results

Equol Stimulates Intracellular ROS Generation in Intact Endothelial Cells

To investigate whether equol stimulates ROS generation, HUVECs were treated with vehicle (0.01% DMSO) or equol (100 nmol/L), and ROS generation was monitored over a 20- to 40-minute assay using lucigenin chemiluminescence. Equol-stimulated ROS production was abrogated by pretreatment with 200 U/mL of SOD (Figure 1A). To confirm the generation of O2−, cells were preincubated with the cell-permeable H2O2 and O2− scavenger Mn (100 μmol/L), PSOD (50 U/mL), or H2O2 metabolizing enzyme catalase (PCAT; 200 U/mL). Equol-mediated increases in lucigenin chemiluminescence were significantly inhibited by Mn, PSOD, and SOD, whereas PCAT failed to inhibit equol-stimulated ROS generation (Figure 1B). To determine whether mitochondria were responsible for equol-induced O2− generation, endothelial cells were pretreated in the absence or presence of the mitochondrial complex I inhibitor rotenone (2 μmol/L) and then challenged with equol. Rotenone abrogated equol stimulated O2− production (Figure 1C), and, furthermore, treatment with equol (100 nmol/L) enhanced cellular fluorescence in HUVECs loaded with
Mitochondrial ROS Generation Is Required for Equol-Induced Kinase and eNOS Phosphorylation

To establish whether mitochondrial $\mathrm{O}_2^-$ plays a role in equol-stimulated eNOS activation, HUVECs were preincubated with rotenone (2 $\mu$mol/L for 30 minutes) and then stimulated acutely with vehicle (0.01% DMSO) or equol (100 nmol/L for 2 minutes) in the continued absence or presence of rotenone. Rotenone blocked the acute phosphorylation of eNOS, Akt, and ERK1/2 by equol, implicating mitochondrial ROS in the upstream activation of kinases.

Mitochondrial Complex I Inhibition Abolishes eNOS-Dependent cGMP Formation

To confirm that activation of kinases and eNOS by mitochondrial $\mathrm{O}_2^-$ influences endothelial NO production, effects of rotenone on equol-induced intracellular cGMP accumulation were measured in HUVECs preincubated with an eNOS inhibitor (N$^\mathrm{G}$-nitro-L-arginine ester; 100 $\mu$mol/L) or rotenone (2 $\mu$mol/L) and then stimulated for 2 minutes with equol (100 nmol/L) in the continued absence or presence of rotenone. N$^\mathrm{G}$-Nitro-L-arginine ester prevented equol-stimulated intracellular cGMP accumulation in HUVECs, confirming intracellular cGMP as a reliable measure NO production (Figure 3G). Consistent with rotenone-mediated inhibition of ROS production and phosphorylation of eNOS, Akt, and ERK1/2, rotenone abrogated equol-stimulated cGMP levels.

Mitochondrial ROS Generation Occurs Downstream of EGFR Activation

ROS generation is known to occur downstream of EGFR activation and to also potentiate EGFR transactivation. To establish a relationship between equol-induced EGFR activa-
Mitochondrial ROS generation is required for equol-induced kinase and eNOS phosphorylation and NO production. HUVECs were preincubated for 30 minutes in Krebs buffer containing L-arginine (100 μmol/L) in the absence or presence of Mn (100 μmol/L), PSOD (50 U/mL), or PCAT (200 U/mL) before acute stimulation with vehicle (Veh; 0.01% DMSO) or equol (100 nmol/L, 2 minutes) in the continued absence or presence of the inhibitors. A through C, Cell lysates were immunoblotted for phosphorylated (p~)eNOS, p~Akt, and p~ERK1/2. Representative immunoblots are shown with densitometric analyses of results from 4 to 5 different cultures summarized in D through F. Mean±SEM of measurements in cultures from 4 to 5 different donors; *P<0.05, **P<0.01 vs vehicle alone; #P<0.05, ##P<0.01 vs vehicle.

Figure 2. Inhibition of ROS generation abrogates equol-stimulated eNOS and Akt phosphorylation. HUVECs were preincubated for 30 minutes in Krebs buffer containing L-arginine (100 μmol/L) in the absence or presence of Mn (100 μmol/L), PSOD (50 U/mL), or PCAT (200 U/mL) before acute stimulation with vehicle (Veh; 0.01% DMSO) or equol (100 nmol/L, 2 minutes) in the continued absence or presence of the inhibitors. A through C, Cell lysates were immunoblotted for phosphorylated (p~)eNOS, p~Akt, and p~ERK1/2. Representative immunoblots are shown with densitometric analyses of results from 4 to 5 different cultures summarized in D through F. Mean±SEM of measurements in cultures from 4 to 5 different donors; *P<0.05 vs vehicle alone; #P<0.05, ##P<0.01 vs vehicle.

Figure 3. Mitochondrial ROS generation is required for equol-induced kinase and eNOS phosphorylation and NO production. HUVECs were preincubated for 30 minutes in Krebs buffer containing L-arginine (100 μmol/L) in the absence or presence of L-NAME or rotenone (Rot; 2 μmol/L) before acute stimulation with equol (100 nmol/L, 2 minutes) in the continued absence or presence of rotenone. Cell lysates were immunoblotted for phosphorylated (p~)eNOS (A), p~Akt (B), and p~ERK1/2 (C) and densitometric analyses are shown in D to E. Mean±SEM of measurements in cultures from 4 to 5 different donors; *P<0.05, **P<0.01 vs vehicle (0.1% DMSO); #P<0.05, ##P<0.01 vs vehicle.

G, HUVECs were preincubated for 30 minutes in Krebs buffer containing L-arginine (100 μmol/L) in the absence or presence of Nomega;-nitro-L-arginine ester (100 μmol/L) or rotenone (2 μmol/L) and then treated with vehicle (Veh; 0.01% DMSO) or equol (100 nmol/L, 2 minutes). Intracellular accumulation of cGMP in HUVEC monolayers was expressed as percentage of change versus vehicle. Mean±SD of triplicate measurements in HUVEC cultures derived from 2 different donors; *P<0.05 vs vehicle; #P<0.05 vs equol.
because equol is structurally similar to estrogen, we hypothesized a role for GPR30 in Akt and ERK1/2 activation involving G protein–linked EGFR transactivation. Pretreatment of HUVECs with the G-protein inhibitor pertussis toxin (100 ng/mL) for 30 minutes blocked equol-stimulated phosphorylation of ERK1/2, Akt, and eNOS (Figure 2A and 2B). A consistent feature of equol-stimulated mitochondrial ROS production (Figure 5C), confirming that F-actin may provide a link between EGFR activation and mitochondrial ROS generation.

**GPR30-Linked Transactivation of EGFR Mediates ERK1/2, Akt, and eNOS Activation**

Estradiol binds GPR30 to stimulate kinase activity, and, because equol is structurally similar to estrogen, we hypothesized a role for GPR30 in Akt and ERK1/2 activation involving G protein–linked EGFR transactivation. Pretreatment of HUVECs with the G-protein inhibitor pertussis toxin (100 ng/mL) or the EGFR kinase inhibitor (AG-1478, 5 μmol/L) for 30 minutes blocked equol-stimulated phosphorylation of ERK1/2, Akt, and eNOS (Figure 6A and 6D). A consistent feature of EGFR transactivation in GPR30 signaling is the recruitment and activation of the protein tyrosine kinase c-Src. Thus, HUVECs were preincubated HUVECs for 30 minutes with a c-Src inhibitor (PP2; 10 μmol/L) and then treated acutely for 2 minutes with equol (100 nmol/L). As shown in Figure 6C and 6F, PP2 blocked equol-stimulated eNOS phosphorylation and significantly attenuated ERK1/2 and Akt phosphorylation. Densitometric analysis of phosphorylated Akt and phosphorylated ERK1/2 is summarized in Figure S3.

**Discussion**

In humans consuming a soy-rich diet, plasma concentrations of equol range between 1 and 100 nmol/L, depending on "equol producer" status. Because equol producers appear to have improved vascular function, it seems likely that the beneficial impact of soy isoflavones on blood pressure and lipid profiles may be influenced by the ability of subjects to metabolize dietary daidzein. Our findings suggest that, in fetal endothelial cells, equol increases mitochondrial ROS, which act as second messengers to induce the rapid stimulation of Akt, ERK1/2, and eNOS activity.

We have obtained novel insights into the cellular mechanisms linking equol-stimulated mitochondrial ROS with activation of eNOS and NO production in endothelial cells. The involvement of ROS in the activation eNOS and upstream kinases was established by observing that inhibition of ROS generation with scavengers of O$_2^-$, but not H$_2$O$_2$ (Figure 1B), abrogated equol-stimulated Akt and eNOS phosphorylation (Figure 2A and 2B). A surprising feature of equol-mediated signaling in endothelial cells is that, although this isoflavone has antioxidant properties in endothelial cells, it also increases mitochondrial ROS, which act as second messengers to induce the rapid stimulation of Akt, ERK1/2, and eNOS activity.
Figure 6. GPR30-linked transactivation of EGFR mediates ERK1/2, Akt, and eNOS activation. HUVECs were preincubated for 30 minutes in Krebs buffer containing L-arginine (100 μmol/L) in the absence or presence of pertussis toxin (PTX; 100 ng/mL), EGFR tyrosine kinase inhibitor AG-1478 (AG; 5 μmol/L), or Src kinase inhibitor (PP2; 10 μmol/L) before acute stimulation with equol (Eq; 100 nmol/L, 2 minutes) in the continued absence or presence of inhibitors. Representative immunoblots for phosphorylated (p-eNOS, p-Akt, and p-ERK1/2 (relative to α-tubulin) are shown in A through C, with densitometric analyses for p-eNOS summarized in D through F. Mean±SEM of measurements in cultures derived from 4 to 5 different donors; *P<0.05 and **P<0.01 vs vehicle; #P<0.05 and ##P<0.01 vs equol.

diseases associated with sustained oxidative stress, under physiological conditions ROS can act as “second messengers” in the regulation of redox-sensitive kinases and transcription factors.25–28

Previous studies reported that activation of eNOS by structurally related polyphenols involves ROS-mediated activation of Akt39,40; however, the intracellular sources and species of ROS were not determined. Mitochondria and NADPH oxidase represent 2 major sources of endothelial ROS generation.28 Notably, rapid stimulation of ROS generation in endothelial cells by 17β-estradiol is inhibited by rotenone but unaffected by inhibitors of NADPH oxidase.35 These studies, together with our present findings, strongly suggest that equol acutely stimulates mitochondrial O2− generation. Because equol-induced ROS generation was completely inhibited by rotenone and equol-enhanced MitoSOX Red fluorescence, it seems unlikely that Nox2 and Nox4, localized predominantly to the plasma membrane and endoplasmic reticulum,31,42 modulated eNOS activity. In endothelial cells, NADPH oxidase can also generate extracellular O2−, which, in turn, may affect intracellular signaling pathways by entering cells through membrane chloride channels.43 In this context, estrogen downregulates NADPH oxidase subunit expression in endothelial cells after 8 hours,44 and equol rapidly inhibits NADPH oxidase activity in macrophages.45

Mitochondria generate ROS via respiratory complexes I and III; however, ROS generation via complex III may play a key role in modulating cytosolic signaling pathways.46 Inhibition of mitochondrial ROS generation in active cells by rotenone suggests that cells were in state 3. Although elevation of intracellular Ca2+ results in mitochondrial Ca2+ loading and ROS generation,47 we reported previously that genistein, daidzein, and equol fail to elicit Ca2+ transients in human endothelial cells,44 suggesting an alternate mechanism for isoflavone-stimulated ROS generation.

Our findings suggest that equol-induced mitochondrial ROS and eNOS activation may be mediated by GPR30-linked transactivation of the EGFR. Treatment with pertussis toxin or AG-1478 abolished phosphorylation of eNOS and the upstream kinases Akt and ERK1/2, with ERK1/2 activity dependent on c-Src activation (Figure 6). Similarly, treatment with AG-1478 inhibited mitochondrial ROS production (Figure 4), indicating that mitochondrial ROS generation occurs downstream of EGFR activation and is unlikely to be attributed to direct binding of equol to the mitochondrial respiratory complexes. EGFR-induced PI3K activation has been suggested previously to mediate mitochondrial ROS production via alterations in mitochondrial ATP-activated potassium channel activity.32 In contrast, our data indicate that kinase activation occurs downstream of mitochondrial ROS production. Several studies have reported that ROS potentiate EGFR transactivation and, thus, kinase activation.33,48 Furthermore, PI3K/ Akt and ERK1/2 kinase pathways are redox sensitive, potentially enabling kinase activation by equol-induced mitochondrial ROS generation.

To our knowledge, we report the first evidence that the isoflavone equol induces rapid alterations in cytoskeletal F-actin distribution (Figure 5). We propose that the mechanism linking EGFR activation and mitochondrial ROS production involves equol-induced alterations in F-actin distribution, because disruption of the cytoskeleton inhibits equol-stimulated mitochondrial ROS generation (Figure 4B). It is unlikely that our findings reflect an artificial disruption of mitochondrial integrity by cytochalasin-D, because previous studies have demonstrated that mitochondria retain their ability to respond to mitochondrial inhibitors, such as antimycin A.34 Recent findings indicate that F-actin may directly bind to the EGFR49 and partition EGFR
receptors to enhance receptor dimerization, which could, in turn, potentiate mitochondrial ROS and kinase activation. The present study highlights a potential protective role for equol in cardiovascular disease. We propose that equol and other isoflavones evoke mitochondrial \( \mathrm{O}_2^- \) generation in endothelial cells, leading to transactivation of the EGFR; activation of e-Src, ERK1/2, PI3K/Akt, and eNOS; and rapid NO release (please see Figure S4).

**Perspectives**

In view of the importance of the developmental origins of health and disease, our study provides novel insights into the mechanisms by which isoflavones acutely regulate eNOS activity, as well as eNOS mRNA and protein expression (please see Figure S5) in fetal endothelial cells. It is worth noting that exposure to dietary soy during fetal development and early life may reduce the susceptibility to cardiovascular disease and obesity in adulthood. By influencing developmental plasticity in utero and in postnatal development, isoflavones may not only alter the expression of genes encoding eNOS but also others associated with metabolism and antioxidant defenses. Thus, based on our previous studies in rodents in vivo and the present findings in fetal endothelial cells, equal and other isoflavones may improve endothelial function and lower blood pressure in the adult via fetal programming.

**Acknowledgments**

We gratefully acknowledge the midwives of St Thomas’ Hospital labor ward and thank Dr Vladimir Smetkov (Division of Ashama, Allergy and Lung Biology, King’s College London, London, United Kingdom) for assistance in fluorescence measurements of mitochondrial ROS generation.

**Sources of Funding**

This work was supported in part by the Biotechnology and Biological Sciences Research Council (BBS/S/K/2004/11207). British Heart Foundation (FS/99075), Heart Research United Kingdom (RG2542 and RG2588), and European Union Cooperation in Science and Technology (COST) Action B35.

**Disclosures**

None.

**References**


Equol-Stimulated Mitochondrial Reactive Oxygen Species Activate Endothelial Nitric Oxide Synthase and Redox Signaling in Endothelial Cells: Roles for F-Actin and GPR30

David J. Rowlands, Sarah Chapple, Richard C.M. Siow and Giovanni E. Mann

_Hypertension_. 2011;57:833-840; originally published online February 7, 2011;
doi: 10.1161/HYPERTENSIONAHA.110.162198
_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/57/4/833

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2011/02/04/HYPERTENSIONAHA.110.162198.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Hypertension_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Hypertension_ is online at:
http://hyper.ahajournals.org//subscriptions/
Equol-stimulated mitochondrial reactive oxygen species activate eNOS and redox signaling in endothelial cells: Roles for F-actin and GPR30

David J. Rowlands†, Sarah Chapple‡, Richard C.M. Siow, and Giovanni E Mann*
Cardiovascular Division, British Heart Foundation Centre of Research Excellence, School of Medicine, King’s College London, 150 Stamford Street, London SE1 9NH, U.K.

†Contributed equally to this work

Address correspondence to:
Prof G.E. Mann, Cardiovascular Division, BHF Centre of Research Excellence, School of Medicine, King’s College London, 150 Stamford Street, London SE1 9NH, UK
Email: giovanni.mann@kcl.ac.uk

Online Data Supplement - Extended Materials and Methods

Materials
Equol was from Apin Chemicals Ltd (UK); MnTMPyP and AG1478 from Calbiochem (USA); L-NAME, IBMX, SOD, PEG-SOD, PEG-CAT, rotenone and pertussis toxin from Sigma-Aldrich (UK). Monoclonal antibodies against eNOS and secondary antibodies were from Santa Cruz Biotechnology (USA), α-tubulin from Chemicon (USA), antibodies against eNOS-Ser1177, dually phosphorylated (threonine 183 and tyrosine 185) ERK1/2 and Akt-Ser473 from Cell Signaling (UK). ECL reagent was from Amersham (UK); MitoSox Red from Invitrogen Molecular Probes (USA); Rhodamine-phalloidin and Hoechst reagent from Fluka (UK); cGMP ELISA from Cayman Chemicals (USA).

Human Umbilical Vein Endothelial Cell Culture
Umbilical cords were obtained with informed patient consent and approval from the St. Thomas’ Hospital Ethics Research Committee. All experiments were performed with passage 3 HUVEC, and an endothelial phenotype was confirmed by a characteristic cobblestone morphology and positive immunostaining for von Willebrand factor (data not shown).1-3

Immunoblotting
HUVEC (passage 3) were equilibrated in low serum (1% FCS) M199, preincubated in Krebs buffer containing L-arginine (100µM) for 30 min and then treated for 2 min with equol (100nmol/L) or vehicle (0.01% DMSO). In other experiments, cells were pretreated for 30 min in the absence or presence of SOD (200U/mL), PSOD (50U/mL), PCAT (200U/mL), rotenone (2µmol/L), pertussis toxin (100ng/mL), AG-1478 (5µmol/L, EGFR tyrosine kinase inhibitor) or 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo [3,4-d] pyrimidine (10µmol/L, PP2, c-Src kinase inhibitor) and then challenged acutely for 2 min with equol (100nmol/L). Reactions were stopped with ice-cold PBS, cell lysates separated by SDS-PAGE, probed with specific antibodies, protein bands detected by ECL and densitometric analyses performed using Image J software (National Institute of Health, USA).2
Quantitative RT-PCR

HUVEC were stimulated with equol (100 nmol/L) for 6h, cells lysed and RNA purified using the Macherey-Nagel RNA isolation kit, quantified and reverse-transcribed using a QuantiTect RT kit (Qiagen). eNOS gene expression was analyzed using a quantitative RT-PCR system (Corbett Rotor gene) and mRNA levels were normalized to the geometric mean of three stable reference genes (ribosomal protein L13a (RPL13A), succinate dehydrogenase (SDHA), β2-microglobulin (β2M)).

Primer sequences were:

eNOS: 5’-GCATCACCGAGAAGAGACT-3’ and 5’-TTCACCTGCTTCGCCACAC-3’
RPL13A: 5’-GAGGCCCTACCCTTCC-3’ and 5’-AACACCTTGAGACGGTCCAG-3’
SDHA: 5’-AGAAGCCCTTTGAGGAGCA-3’ and 5’-CGATTACGGGTCTATATTCCAGA-3’
β2M: 5’-TTCTGGCCTGGAGGCTATC-3’ and 5’-TCAGGAATTTGACCTTCCATTCC-3’

Intracellular cGMP Accumulation as an Index of NO Production

Confluent cell monolayers were preincubated for 30min with Krebs buffer containing L-arginine (100µmol/L) and the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX, 0.5 mmol/L) in the absence or presence of L-NAME (100µmol/L) or rotenone (2µmol/L). Cells were then stimulated acutely for 2min with equol (100nM) or vehicle (Veh, 0.01% DMSO) in the presence of IBMX (0.5mM) and L-arginine (100µmol/L) and continued absence or presence of L-NAME (100µmol/L) or rotenone (2µmol/L). Basal and stimulated intracellular cGMP levels were determined by ELISA, with inhibition of cGMP accumulation by L-NAME serving as an index of NO production.

References

Figure S1. Concentration-dependent activation of eNOS and upstream kinases by the isoflavone equol. HUVEC were equilibrated in low serum (1% FCS) M199 for 4 h and then incubated in Krebs buffer containing L-arginine (100μmol/L) and stimulated for 2 min with equol (10^{-9}–10^{-5} M) or vehicle (Veh, 0.01% DMSO). Cell lysates were immunoblotted for p-eNOS, p-Akt and p-ERK1/2. (A) Representative immunoblot of eNOS phosphorylation. (B-D) Densitometric analyses of p-eNOS, p-Akt and p-ERK1/2 in HUVEC stimulated acutely with equol vs. vehicle. Mean ± S.E.M. of values in cultures from 3-4 different donors, *P<0.05 relative to vehicle.
Figure S2. Time-dependent activation of eNOS phosphorylation by the isoflavone equol. HUVEC were equilibrated in low serum (1% FCS) M199 for 4 h and then incubated in Krebs buffer containing L-arginine (100 μmol/L) and stimulated for 2 – 30 min with equol (100 nmol/L) or vehicle (Veh, 0.01% DMSO). Cell lysates were immunoblotted for phosphorylated eNOS-Ser1177 and analyzed by densitometry relative to α-tubulin. Mean ± S.E.M. of values in cultures from 3-4 different donors, *P<0.05, **P<0.01 relative to vehicle.
Figure S3. Densitometric analysis of equol stimulated Akt and ERK1/2 phosphorylation in fetal endothelial cells - effects of inhibitors of G-proteins, EGFR kinase and c-Src kinase. HUVEC were pre-equilibrated in low serum (1% FCS) M199 for 4 h and then pre-incubated for 30 min with the G-protein inhibitor (pertussis toxin, PTX, 100 ng/mL), EGFR kinase inhibitor (AG-1478, AG, 5µmol/L) or Src kinase inhibitor (PP2, 10 μmol/L) prior to acute stimulation with equol (Eq 100 nmol/L) for 2 min in the absence or presence the inhibitors. Membranes were immunoblotted for p~Akt (panels A-C) and p~ERK1/2 (panels D-F) vs. α-tubulin. Mean ± S.E.M of measurements in different cell cultures obtained from 4 different donors, *P<0.05, **P<0.01 vs. vehicle (Veh, 0.01% DMSO) and #P<0.05, ##P<0.01 vs. equol in the absence of inhibitors.
**Figure S4.** Proposed mechanisms underlying acute activation of eNOS by equol and other dietary isoflavones. Equol and other dietary isoflavones stimulate kinase activation and subsequent eNOS phosphorylation and NO production via mitochondria-derived ROS. Kinase activation may be dependent on GPR30/epidermal growth factor receptor (EGFR) transactivation as previously described.\textsuperscript{21,24} EGFR activation leads to ERK1/2 activation via c-Src and mitochondrial-ROS potentiated Akt activation via PI3-K. Notably, inhibition of mitochondrial ROS generation with rotenone or depolymerization of F-actin abrogates equol stimulated activation of Akt, eNOS and NO production in human endothelial cells.
**Figure S5.** Equol upregulates eNOS protein and mRNA expression in fetal endothelial cells. HUVEC were pre-incubated in low serum (1% FCS) M199 for 4 h and then treated with M199 (1% FCS) containing equol (Eq, 100 nmol/L) or vehicle (Veh, 0.01% DMSO) for 4–24 h. (A–B), effects of equol on eNOS protein expression in a representative immunoblot and densitometric analysis of eNOS/α-tubulin ratio. (C) eNOS mRNA expression after 6 h treatment with equol (Eq, 100 nmol/L, filled squares) vs. vehicle (Veh, 0.01% DMSO, unfilled squares), expressed as mean copy number relative to the Geomean of three house-keeper genes (see Supplemental Materials and Methods). Means ± S.E.M., n = 5–6 different cell cultures from 5-6 different donors, *P<0.05, vs. vehicle.