Review

Activation of endothelial nitric oxide synthase by dietary isoflavones: Role of NO in Nrf2-mediated antioxidant gene expression

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Abstract

The endothelium plays a key role in the maintenance of vascular homeostasis, and increased oxidative stress in vascular disease leads to reduced nitric oxide bioavailability and impaired endothelium-dependent relaxation of resistance vessels. Although epidemiological evidence suggests that diets containing high amounts of natural antioxidants afford protection against coronary heart disease (CHD), antioxidant supplementation trials have largely reported only marginal health benefits. There is controversy concerning the cardiovascular benefits of prolonged estrogen/progestin or soy isoflavone therapy for postmenopausal women and patients with an increased risk of CHD. Research on the potential health benefits of soy isoflavones and other polyphenols contained in red wine, green and black tea and dark chocolate developed rapidly during the 1990’s, and recent clinical trials and studies in animal models and cultured endothelial cells provide important and novel insights into the mechanisms by which dietary polyphenols afford protection against oxidative stress. In this review, we highlight that NO and reactive oxygen radicals may mediate dietary polyphenol induced activation of Nrf2, which in turn triggers antioxidant response element (ARE) driven transcription of phase II detoxifying and antioxidant defense enzymes in vascular cells.

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Keywords: Endothelial cells; Nitric oxide; Endothelial nitric oxide synthase; Caveolin-1; Heat shock protein 90; Soy isoflavones; Genistein; Daidzein; Equol; Red wine polyphenols; Tea polyphenols; Phosphoinositol 3-kinase; Extracellular signal-regulated kinase; cAMP; CREB; Nrf2; Antioxidant response element; Electrophile response element; Heme oxygenase-1; Estrogen receptors; Estrogen response element; Oxidative stress; Redox signaling

1. Introduction

Nutrient–gene interactions have gained wide acceptance in the ‘post-genomic’ era, and nutrigenomic approaches are yielding insights at genomic, proteomic and metabolomic levels to evaluate the therapeutic potential of diets in the prevention and/or treatment of cardiovascular diseases [1]. Although diets containing natural antioxidants appear to afford protection against coronary heart disease (CHD), antioxidant supplementation provides only marginal health benefits [2], as evidenced by the Heart Outcomes Prevention Evaluation trial and its failure to show benefits of vitamin E supplementation in patients at high risk of CHD [3].

Polyphenols are organic compounds synthesized by plants, including tannins, lignans and flavonoids. Isoflavones are flavonoid compounds with both antioxidant and estrogenic properties, such as the soybean isoflavones genistein and daidzein which can behave as estrogen mimics [4]. The current interest in dietary soy isoflavones (‘phytoestrogens’) [4–9] is based on epidemiological evidence that an increased intake of soy isoflavones is associated with a lower incidence of CHD [9,10]. Soybean proteins and products contain significant amounts of the isoflavones genistein and daidzein, either in an unconjugated aglycone form or different glycoside conjugates [4]. Plasma concentrations of genistein are as low as 40 nM in humans consuming Westernized diets but can reach 4 μM in Japanese consuming a traditional soybean rich diet [4,10,11].

Polyphenols are also abundant in grape seeds and skins, and red wine is rich in proanthocyanidins (e.g. oligomers of
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<td>Healthy men (20–51 y), premenopausal women (29–33 y): acute brachial artery infusion of genistein or daidzein (52–60 y); 6 months genistein or placebo</td>
<td>Genistein (10–300 nM, 6 min), but not daidzein, ↑ forearm blood flow in men + premenopausal women; blunted by l-NMMA but significant endothelium-independent component of relaxation</td>
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<td>Genistein</td>
<td>Randomized, double-blind, placebo-controlled: healthy postmenopausal women (52–60 y); E&lt;sub&gt;2&lt;/sub&gt; norethisterone, genistein or placebo for 1 y</td>
<td>FMD brachial artery by high-resolution ultrasound: genistein ↑ brachial artery diameter &gt;100%; plasma levels of NOx ↑ (22 vs 44 μM), ET-1 ↓ (14 vs 7 pg/ml); plasma genistein levels ↑ (0.07 vs 1.2 μM) after 6 months</td>
<td>[45]</td>
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<td>Randomized, placebo-controlled, cross-over: healthy perimenopausal/postmenopausal women; isolavones daily for 5–10 weeks</td>
<td>Improved systemic arterial compliance assessed by ↑ pulse wave velocity; ↑ systolic (125 vs 121 mm Hg) and diastolic (78 vs 75 mm Hg) blood pressure; no change plasma total cholesterol, LDL, triglyceride or glucose</td>
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<td>Randomized, double-blind, placebo-controlled: healthy men/postmenopausal women (50–70 y); soy isolate powder vs casein placebo 3 months</td>
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<td>[177]</td>
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<td>Soy isoflavones</td>
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<td>Soy isoflavones no effect on arterial blood pressure or plasma lipid profile; systemic arterial compliance improved; ↑ brachial artery dilation to ACh similar in isolavones vs placebo cohort; note patients’ variable hormone status</td>
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<td>Soy isoflavones</td>
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<td>Soy isoflavones</td>
<td>Randomized, double-blind, placebo-controlled, cross-over: healthy postmenopausal women (mean 57 y), 8 week cereal bars (± isolavones)</td>
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<td>Soy isoflavones</td>
<td>Randomized, double-blind, placebo-controlled, cross-over: hypertensive subjects (26 men and 15 postmenopausal women (30–75 y); soy cereal 6 months</td>
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<td>Black tea 900 ml freeze-dried tea daily for 4 weeks; plasma catechin ↑ from 25 to 35 ng/ml</td>
<td>[65]</td>
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<td>Flavanol-rich</td>
<td>Randomized, double-blind, placebo-controlled: healthy women/men (21–55 y), 2 weeks daily dark chocolate, low vs high flavonoid content</td>
<td>High procyanidin dark chocolate bars ↑ plasma epicatechin from -25 to 200 nM; FMD responses of brachial artery ↑ from 10% to 12%; no changes in plasma LDL oxidation or 8-isoprostanes levels</td>
<td>[73]</td>
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<td>Flavanol-rich</td>
<td>Randomized, single-blind, cross-over: healthy subjects (12 men/5 women, 24–32 y), 2 weeks daily dark chocolate, low vs high flavonoid content</td>
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<td>Flavanol-rich</td>
<td>Randomized trials: healthy (7 men, 8 women, 34 y) + essential hypertension patients (10 men/10 women, 44 y, 7 d flavanol cocoa)</td>
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<td>Flavanol-rich cocoa</td>
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<td>Flavanol-rich cocoa</td>
<td>Randomized, double-blind, cross-over: smokers, cocoa drink (high vs low flavanol)</td>
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<td>Flavanol-rich cocoa</td>
<td>Randomized, double-blind, cross-over: healthy males (25–32 y) consuming cocoa (high vs low flavanol content)</td>
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<td><strong>Abbreviations</strong>: FMD, Flow-mediated dilation; eNOS, endothelial nitric oxide synthase; l-NMMA, N&lt;sup&gt;ω&lt;/sup&gt;-monomethyl-L-arginine (NOS inhibitor); l-NNAME, nitro-L-arginine methyl ester; NOx, plasma nitric oxide metabolites; ET-1, endothelin-1; HRT, hormone replacement therapy.</td>
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catechin/epicatechin) [12]. Although resveratrol is found in the skin of grapes, its low abundance in red wine may limit its benefits for cardiovascular health. Nevertheless, resveratrol has been shown to extend the lifespan of mice on a high calorie diet [13]. Other rich sources of polyphenols are beans, apples, cranberries, hawthorn extract, green and black tea and dark chocolate [14–16]. As with dietary soy isoflavones, epidemiological studies attribute the lower incidence of CHD in France to an increased consumption of red wine rich in proanthocyanidins [17–19]. However, other factors may contribute to the ‘French’ paradox that populations with risk factors similar to those in the United States and northern Europe have significantly lower mortality due CHD [20]. Bioavailability of dietary polyphenols will be affected by rapid metabolism in the gastrointestinal tract and rates of intestinal absorption, with isoflavones absorbed more rapidly than catechins or proanthocyanidins [14,15].

Although randomized hormone replacement trials such as the Heart and Estrogen/progestin Replacement Study (HERS) and the Women’s Health Initiative Study (WHI) reported a lack of benefit of combined estrogen/progestin therapy for cardiovascular disease [21,22], it is worth emphasizing that the conjugated equine estrogens used in these trials may not mimic the vascular actions of estradiol [23]. Thus research into alternatives for hormone replacement therapy has focused primarily on the potential cardiovascular benefits of natural polyphenolic compounds. The isoflavones genistein and daidzein are structurally similar to estrogen [4], and their preferential affinity for estrogen receptor (ER) β [24] provided a basis for evaluating the efficacy these isoflavones as modulators of vascular function without the undesired effects on breast and uterine tissue associated with prolonged estrogen therapy [21,22]. An increased intake of isoflavones by postmenopausal women in the Framingham Offspring Study highlighted a favorable cardiovascular risk profile [25], and the US Food and Drug Administration initially endorsed the health benefits of soy products [26]. However, in 2006 nutritional guidelines were revised by the American Heart Association [27], who concluded that the evidence for cardiovascular protection afforded by isoflavones is minimal and that health benefits of soy protein could be due to the high content of polyunsaturated fats (and low saturated fats), fiber, vitamins, and minerals [27].

Numerous clinical trials have examined the effects of isoflavone supplementation on vascular reactivity and arterial blood pressure in subjects who were elderly, overweight and/or smokers, potentially limiting interpretation of the cardiovascular health benefits of isoflavones. In addition, the outcome of several randomized trials with small cohorts of healthy subjects, hypertensive patients or postmenopausal women may be confounded by non-compliance with dietary guidelines, anti-hypertensive therapy and/or the variety of isoflavone formulations, their metabolic fate and bioavailability.

We have reviewed the evidence that soy isoflavones and polyphenols contained in red wine, green and black tea and dark chocolate influence vascular reactivity by targeting endothelial nitric oxide synthase (eNOS) and redox sensitive gene expression. Increased endothelium-dependent NO generation in response to laminar shear stress, vasoactive agonists and dietary polyphenols may modulate cellular sensor(s) for oxidative stress [28,29] and thereby increase NO bioavailability. Under conditions of increased oxidative or nitrosative stress, NO can react with superoxide anions (O$_2^-$) to form peroxynitrite [30]. NO and peroxynitrite enhance nuclear accumulation of Nrf2 (nuclear factor erythroid 2-related factor), a redox sensitive basic leucine-zipper transcription factor involved in antioxidant response element (ARE or electrophile response element, EpRE) dependent gene expression [31–34]. Under basal conditions, Nrf2 interacts with a cytosolic repressor protein Keap1 (Kelch ECH associating protein) limiting Nrf2 mediated gene expression [31]. In cells exposed to oxidative or xenobiotic stress, Nrf2 is released from Keap1 and translocates to the nucleus, where it activates ARE dependent transcription of phase II and antioxidant defense enzymes, such as NAD(P)H:quinone oxidoreductase, glutathione-S-transferase, glutathione peroxidase and heme oxygenase-1 [31–35].

2. Vascular actions of soy isoflavones and other polyphenols in vivo

In two clinical studies, isoflavones have been shown to acutely modulate vascular reactivity in healthy postmenopausal women and male subjects in vivo [36,37]. Infusion of genistein or dihydroequol into the brachial artery, at concentrations achieved in populations consuming an isoflavone rich diet [4,11], evoked concentration- and endothelium-dependent increases in forearm blood flow (see Table 1) [36,37]. As a significant component of genistein mediated dilation was insensitive to L-NMMA [38,39], increases in plasma nitrite/nitrate levels [40,41–43], increases plasma nitrite/nitrate levels [45–48] and decreases plasma endothelin-1 levels [43,45,46]. However, some clinical studies have reported negligible effects of isoflavone supplementation on plasma endothelin-1 and/or nitrite levels [49,50], and the evidence for an improvement in plasma lipid profiles remains conflicting [21,48,51,52]. Several randomized, double-blind, placebo-controlled trials report cardiovascular benefits of longer-term (2–12 months) supplementation with genistein, tetrahydrodaidzein or soy isoflavones [42,44–46,48,53]. The consensus of such studies in healthy postmenopausal women is that isoflavone supplementation increases brachial artery flow-mediated dilation and improves systemic arterial compliance (Table 1) [41,42,44]. Despite numerous clinical studies, there is still controversy whether isoflavone supplementation
lowers arterial blood pressure in vivo [42,44,53–55]. It is possible that vascular responses to isoflavone metabolites [54,55] may be limited to individuals capable of metabolizing ingested daidzein to equol [5,56,57]. In summary, the benefits of genistein supplementation on endothelial function have largely been observed in humans and animal models with mild to moderate hypertension [41,58–60].

We recently reported that feeding a soy protein diet rich in genistein and daidzein, known to interact with estrogen receptors [24], increases mRNA levels of eNOS and antioxidant enzymes in aged male rats [6]. In contrast, an isoflavone deficient diet fed from conception throughout adult life was associated with decreased GSH concentrations and mRNA levels for eNOS, MnSOD and cytochrome c oxidase, impaired endothelium-dependent relaxation and increased blood pressure in vivo (Table 2). Since a balanced isoflavone diet fed to rats from conception to 6 months of age has only marginal benefits for endothelial function [61], isoflavone supplementation may only restore vascular reactivity in aged animals or subjects with cardiovascular disease [6,62]. Chronic feeding of stroke-prone hypertensive rats with the flavonoid quercetin partially reverses elevated blood pressure and impaired endothelium-dependent relaxation in these animals [63]. The molecular mechanisms by which polyphenols diminish oxidative stress and reduce blood pressure in vivo remain to be elucidated. Nevertheless, vascular protection afforded by soy isoflavone diets may be related to an increased activity and/or expression of eNOS and NO bioavailability [9,64].

Randomized clinical studies in healthy human volunteers, smokers and patients with coronary artery disease have shown that consumption of black and green tea polyphenols or flavanol-rich chocolate/cocoa enhances flow-mediated brachial artery dilation within 1–24 h (Table 1) [65–71]. Clinical studies with healthy volunteers report that ingestion of flavanol-rich chocolate/cocoa results in a L-NAME sensitive peripheral vasodilation [72], increased insulin sensitivity and reduced blood pressure [70]. Moreover, patients with essential
Soy isoflavones

Genistin, daidzein, equol

HUVEC

Isoflavones (0.1–100 nM, 0.5–2 min) rapidly ↑ Akt/eNOS Ser1177 phosphorylation, ↑ NO release; by PKA inhibitor (889, 10 μM, but unaffected by inhibitors of PKA/Akt or ERK1/2); ↑ adenyl cyclase/PKA; CREB activity (5–10 μM, 15–30 min); insensitive to inhibition by ICI 182,780
[8]

Genistein, daidzein

BAEC

Isoflavones (0.1–90 nM, 10 min) ↑ eNOS Ser1177 phosphorylation, ↑ NO release; by PKA inhibitor (889, 10 μM, but unaffected by inhibitors of PKA/Akt or ERK1/2); ↑ adenyl cyclase/PKA; CREB activity (5–10 μM, 15–30 min); insensitive to inhibition by ICI 182,780
[123,124]

Genistin, daidzein

EA.hy926 cells

High isoflavone concentrations (10 μM, 20 h) ↑ eNOS luciferase reporter gene expression; ↑ cGMP production from cAMP-agarose after 48–96 h
[126]

Genistin

Rat aorta and pulmonary artery

Isolierung (3–38 μM) ↓ relaxation of precontracted aortic rings; ↓ by endothelium denudation or t-NAME (100 μM); ↓ by pretreatment with the ICI 182,780; 96 h ± inhibitors of PI3K/Akt or ERK1/2; ↓ by pretreatment with the ICI 182,780↓ by endothelium denudation or t-NAME (100 μM); ↓ by pretreatment with the ICI 182,780; 96 h ± inhibitors of PI3K/Akt or ERK1/2; ↓ by pretreatment with the ICI 182,780; 96 h
[40]

Genistein

Rat aorta

Wistar–Kyoto/ShR male rats (genistin 10 mg/kg b.w. daily, 5 weeks); ↓ systolic blood pressure SHR rats; ↑ ACh relaxation/eNOS activity in SHR rings; insensitive to inhibition by ICI 182,780
[59]

Daidzein

Rat aorta, carotid artery

Male rats (daiadzein 0.2 mg/kg per s.c., 7 d); ↓ ACh relaxation of precontracted carotid artery rings; ↑ cGMP (2-10 μM) + nitrite/nitrate; eNOS protein unchanged; ↑ caveolin-1 expression; ICI 182,780 induced relaxation masked dilator action of daidzein
[179,180]

Dihydrodaidzein, trans-

tetrahydro-daidsen, equol

Rat aorta

Isoflavone metabolites (30–300 nM) acutely relax precontracted aortic rings; ↓ by inhibitors of eNOS (t-NAME, 10 μM, soluble guanylyl cyclase (ODQ, 10 μM) but unaffected by indomethacin (10 μM); ↓ relaxation after endothelium denudation
[181]

Equol

Rat aorta

Equol (0.03–3 μM) relaxes precontracted, endothelium-intact aortic rings; ↓ by t-NAME (100 μM)
[8]

Quercetin

Rat aorta

Male SHR and Wistar–Kyoto (WKY) rats (5 weeks old); ↑ blood pressure in SHR rats + ACh mediated relaxation of preconstricted aortic rings; ↓ p47(Src) phosphorylation↑ CREB activity (5–30 min); plasma isoflavone levels not measured
[183]

Soluble wine polyphenols

Rat aorta

Ovariectomized rats fed SoyAct TM (52% genistein, 42% daidzein, 6% glycitein); ↑ ACh mediated relaxation of preconstricted rings; plasma isoflavone levels not measured
[182]

SoyLife 150

Rabbit aorta

Ovariectomized WtLL rats (SoyLife 150, 16 weeks); unaltered CCh relaxation of preconstricted cerebral/basilar artery (10 μM); eNOS mRNA; plasma (ng/ml): genistein (23), glycitein (252), daidzein (182)
[183]

Table 2

Dietary soy isoflavones and polyphenols acutely activate eNOS in cultured endothelial cells and isolated arterial preparations

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<td>Genistin, daidzein, equol</td>
<td>HUVEC</td>
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<td>BAEC</td>
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| Equol                           | Rat aorta                           | Equol (0.03–3 μM) relaxes precontracted, endothelium-intact aortic rings; ↓ by t-NAME (100 μM) | [8]       |
| Quercetin                       | Rat aorta                           | Male SHR and Wistar–Kyoto (WKY) rats (5 weeks old); ↑ blood pressure in SHR rats + ACh mediated relaxation of preconstricted aortic rings; ↓ p47(Src) phosphorylation↑ CREB activity (5–30 min); plasma isoflavone levels not measured | [183]     |
| Soy isoflavones                 | Rat aorta                           | Ovariectomized rats fed SoyAct TM (52% genistein, 42% daidzein, 6% glycitein); ↑ ACh mediated relaxation of preconstricted rings; plasma isoflavone levels not measured | [182]     |
| SoyLife 150                     | Rabbit aorta                        | Ovariectomized WtLL rats (SoyLife 150, 16 weeks); unaltered CCh relaxation of preconstricted cerebral/basilar artery/eNOS mRNA; plasma (ng/ml): genistein (23), glycitein (252), daidzein (182) | [183]     |
hypertension consuming flavanol-rich dark chocolate exhibit increased brachial artery flow-mediated dilation and a significant reduction in 24 h systolic blood pressure [71]. Only a limited number of clinical studies have measured plasma concentrations of nitroso species, 8-isoprostanes, epicatechin and/or epicatechin-7-β-gluronide (Table 1). Although flavanol-rich chocolate/cocoa increases plasma epicatechin concentrations and endothelium-dependent dilation and pure epicatechin mimics these effects [68, 73], other bioactive components in chocolate could contribute to vascular relaxation in vivo. In this context, some studies report only limited relaxation of rat aortic rings [74] and eNOS activation in the EA.hy296 endothelial cell line [75] in response to catechin and epicatechin. Flavanols and green tea polyphenols have been reported to have antioxidant effects in human plasma [76] and modulate redox signaling in cells [77, 78], providing additional mechanisms for increasing NO bioavailability.

3. eNOS as a vascular target of soy isoflavones and other polyphenols

A series of seminal papers in the 1980’s identified endothelium-derived relaxing factor (EDRF) as NO, which is synthesized by eNOS from the cationic amino acid l-arginine in the presence of molecular oxygen and other cofactors [79–81]. As a Ca2+/calmodulin (CaM)-dependent enzyme, eNOS is regulated by its association with the plasmalemmal scaffolding protein caveolin-1 [82, 83], post-translational modification via phosphorylation by protein kinases and an interaction with the molecular chaperone heat shock protein 90 (Hsp90) [84–90]. Tethering of eNOS to caveolin-1 maintains the enzyme in an inactive state, and an elevation in intracellular Ca2+ leads to vasoactive agonists promoting association of CaM with eNOS and dissociation of the enzyme from caveolin-1 [91].

In addition to Ca2+-dependent activation of eNOS, laminar shear stress [87, 92], adenosine [93], β2-adrenoceptor agonist [94], 17β-estradiol [95] and isoflavones [8] stimulate NO production via phosphorylation of eNOS at cytosolic Ca2+ levels, with some studies reporting rapid dissociation of eNOS from caveolin-1 and association with heat shock protein Hsp90 (Fig. 2). The major phosphorylation sites include Ser1177 (or Ser1179 in bovine cells) in the reductase domain and Thr495 and Thr497 in the CaM binding domain. Akt [96], protein kinase A [97], mitogen-activated protein kinases ERK1/2 [98] and p38MAPK [99] have all been reported to phosphorylate Ser1177. Phosphorylation at Thr495 antagonises binding of CaM to eNOS [100] and enzyme activation, and thus a concerted...
phosphorylation at Ser1177 and dephosphorylation of Thr495 occurs in response to different stimuli.

García-Cardena et al. [84] were the first to report an association of eNOS with the molecular chaperone protein Hsp90 in endothelial cells. Hsp90 belongs to a family of molecular chaperones that facilitate activation or correct folding of a wide range of proteins, including nuclear hormone receptors, protein kinases and eNOS [101–103]. Although Hsp90 does not affect eNOS dissociation from caveolin-1 in the absence of Ca²⁺/CaM, it facilitates CaM-mediated release of eNOS from caveolin [88]. Binding of Hsp90 to eNOS is essential for Akt-mediated activation of the enzyme, and importantly Hsp90 functions not only to recruit Akt to eNOS but also prevents proteasomal degradation of phosphoinositide-3-kinase 1 which activates Akt [90]. In the case of vascular endothelial growth factor (VEGF), Hsp90 modulates the transition from an initial Ca²⁺-dependent to a delayed and sustained phosphorylation-dependent activation of eNOS [104].

NO production can be inhibited by disrupting eNOS–Hsp90 association, using geldanamycin or radicicol, which bind to the N-terminal ATP-binding site of Hsp90 [105]. Although radicicol has been reported to inhibit src kinase, it initially inhibits Hsp90, which in turn destabilises src kinase. There are conflicting reports whether geldanamycin affects Akt activity or only the association of Akt with eNOS [90,104]. In the context of vascular oxidative stress, disruption of eNOS-Hsp90 can result in eNOS uncoupling and increased O₂⁻ production [85]. Geldanamycin, but not radicicol, redox cycles to generate O₂⁻ [106,107], however, the low levels of O₂⁻ generated may have a negligible impact on NO production [108]. Studies of Hsp90 interaction with purified tetrahydrobipterin (BH₄)-free eNOS revealed that BH₄ and not Hsp90, inhibits Ca²⁺/CaM-dependent O₂⁻ formation from purified eNOS [109]. Importantly, depletion of BH₄ in bovine aortic endothelial cells does not alter Hsp90 levels or its interaction with eNOS, with increased O₂⁻ generation occurring independent of eNOS Ser1179 phosphorylation. Hsp90 protein levels are diminished in endothelial cells from pre-eclamptic pregnancies [110], which are characterized by an increased placental production of reactive oxygen species [111,112]. However, a recent review highlights that Hsp90 expression is upregulated under conditions of oxidative stress [113].

### 3.1. Acute activation of eNOS by soy isoflavones

The signaling pathways underlying eNOS activation by estrogens have been reviewed extensively [114–117], and co-localization of estrogen receptors (ERα and ERβ) with eNOS in membrane caveolae in endothelial cells provides a functional signaling unit for enzyme activation [118–120]. Hsp90 can interact directly with ERs, enabling 17β-estradiol to rapidly activate eNOS [121]. Although a subpopulation of estrogen-like receptors in the plasma membrane of endothelial cells may also mediate rapid activation of intracellular signaling pathways [8,122], there is no direct evidence that isoflavones interact with plasma membrane ‘estrogen receptors’.

As summarized in Table 2, isoflavones elicit both endothelium-dependent and -independent relaxation of preconstricted arterial rings in vitro, mimicking the acute dilator actions of genistein and dehydroequol in the human forearm vasculature [36,37]. We previously reported that the daidzein metabolite equol causes acute relaxation of rat preconstricted aortic rings [8]. Equol mediated relaxation was inhibited by l-NAME (Fig. 1A–C), whereas the environmental estrogenic pollutant 4-octylphenol and ICI 182,780 (not shown) reduced coronary perfusion pressure in isolated Langendorff hearts (Fig. 1D) via an inhibition of L-type Ca²⁺ channels in vascular smooth muscle (Fig. 1E, [38]).

As summarized in Fig. 2, low nanomolar concentrations of isoflavones rapidly stimulate NO release from cultured endothelial cells [8,123,124]. Our studies in human umbilical vein endothelial cells (HUVEC) established that genistein, daidzein and equol acutely (100 nM, 30 s–2 min) stimulate ERK1/2 and Akt-dependent eNOS phosphorylation at basal cytosolic Ca²⁺ levels. Isoflavone stimulated NO release was unaffected by the ER antagonists ICI 182,780 (Faslodex) and tamoxifen or uncoupling of G-protein receptors [8]. We further established that equol (100 nM) induces a rapid (2 min) association of eNOS with Hsp90 [8]. In contrast, genistein stimulated eNOS phosphorylation in bovine aortic endothelial cells (BAEC) occurs only after 5–10 min, involving activation of PKA but independent of extracellular signal-regulated kinase 1/2 or PI3-kinase [123]. Although genistein does not acutely elevate cAMP levels in HUVEC [8], treatment of BAEC with higher concentrations of genistein (5 μM, 30 min) increases cAMP levels and activates the transcription factor CREB involved in cAMP-mediated gene expression [124]. Thus, genistein stimulated eNOS activation may involve different signaling pathways in human and bovine endothelial cells. In the majority of studies with arterial ring preparations and endothelial cells rapid activation of eNOS by isoflavones is insensitive to inhibition by ICI 182,780 (Table 2). As high micromolar concentrations of genistein are well known to inhibit tyrosine kinase activity [125], caution is warranted in interpreting increased eNOS luciferase reporter activity in EA.hy926 endothelial cell line [126]. It is worth re-emphasizing that physiological plasma concentrations of genistein range between 0.1 nM to 1 μM [11].

### 3.2. Activation of eNOS by red wine polyphenols and flavanols in dark chocolate

Studies with isolated arterial rings have shown that red wine polyphenols and grape seed extracts increase endothelium/NO-dependent relaxation (see Table 2) [74,127–133]. Treatment of cultured endothelial cells with relatively high concentrations of resveratrol (30–100 μM) or red wine
polyphenols increases eNOS mRNA levels and NO synthesis [75,134,135], however, two studies report negligible effects of resveratrol in human endothelial cells [135] and rat aortic rings [136]. Polyphenols contained in green and black tea and dark chocolate also activate eNOS in endothelial cells and augment endothelium-dependent relaxation in isolated arterial ring preparations [68,137–142]. Administration of black and green tea polyphenols for 3 weeks to stroke-prone hypertensive rats was associated with a decrease in systolic blood pressure in vivo [143], suggesting that elevated plasma catechin levels may stimulate endothelium-derived NO synthesis. As summarized in Table 2 and a recent review [144], there are reports that catechin and epicatechin elicit only limited vascular responses [74,75]. As recently reviewed [144], tea polyphenols modulate the activity of various kinases and have been reported to upregulate HO-1 expression in endothelial cells [145].

4. Nitric oxide and peroxynitrite modulate Nrf2/ARE mediated redox signaling

Few studies have addressed the molecular and cellular targets of dietary polyphenols in vascular cells (Table 2). Estrogens and phytoestrogens can act as free radical scavengers [146], however, recent evidence indicates estrogens [147,148], isoflavones [6], red wine and tea polyphenols [75,134,135] and flavanol-rich dark chocolate can increase mRNA and protein levels for eNOS in endothelial cells (see Table 2). Moreover, polyphenols have been implicated in ARE-mediated gene transcription [147,148] and upregulation of MnSOD expression in vascular cells [149].

Induction of antioxidant genes is regulated through a cis-acting ARE element within the regulatory region of target genes [33]. The redox-sensitive basic leucine zipper protein transcription factor Nrf2 is involved in the regulation of many detoxification and antioxidant genes. Nrf2 belongs to the Cap-N-Collar family of transcription factors, forming heterodimers with small Maf proteins, with subsequent binding to ARE leading to transcriptional gene activation [28,31–33,35]. To our knowledge there are no reports suggesting that estrogens or isoflavones induce antioxidant genes via ERα or ERβ mediated transactivation of ARE. Moreover, genistein, daidzein and equol acutely activate eNOS phosphorylation and NO release independent of the classical estrogen receptors (Table 2) [8,123].

An exposure of endothelial cells to donors of NO and/or peroxynitrite leads to adaptive increases in glutathione (GSH) synthesis and Nrf2/ARE leads to transcriptional up-regulation of genes encoding heme oxygenase-1 (HO-1), γ-glutamylcysteine synthetase (γ-GCS) and the l-cystine anionic amino transporter xCT [150–152]. The microsomal enzyme HO-1 metabolizes heme to generate biliverdin/bilirubin and carbon monoxide, which like NO can inhibit platelet aggregation and act as a vasodilator when the bioavailability of NO is limited [153–155]. Biliverdin is subsequently converted by biliverdin reductase to bilirubin, a chain-breaking antioxidant which can scavenge lipid peroxyl radicals [156]. l-cystine transport is a rate-limiting for GSH biosynthesis in endothelial cells exposed to oxidative stress [157,158], providing an adaptive mechanism to reduce oxidative stress associated with lipid peroxidation. Treatment of bovine aortic endothelial cells S-nitrosopenicillamine or SIN-1 (∼0.1–1 mM, 16–18 h) stimulates l-cysteine transport [150], suggesting that donors of NO and peroxynitrite activate Nrf2/ARE mediated expression of xCT transporter. In the same cell type, spermine NONOate causes rapid nuclear translocation of Nrf2 (2–4 h), phosphorylation of ERK1/2, p38 and JNK mitogen-activated protein kinases and increased activity/expression of xCT and HO-1 [151]. Inhibitors of MAPK cascades reduce Nrf2 translocation, implicating MAPK as modulators of NO induced Nrf2/ARE signaling [159–161]. Notably, pretreatment of endothelial cells with the antioxidant N-acetylcysteine only decreases NO stimulated HO-1 expression by <40% [151].

Accumulating evidence suggests that the Keap1-Nrf2 complex constitutes a sensor of oxidative stress involved in triggering ARE mediated gene expression to restore the cellular redox status [28]. If NO were able to S-nitrosylate ‘oxidant sensing’ cysteine residues in the intervening region of Keap 1 [162], this would promote dissociation of Nrf2 from the complex and translocation into the nucleus to initiate ARE mediated gene transcription. In this context, studies in neuroblastoma cells have shown that NO stimulated activation of Nrf2/ARE leads to transcripational up-regulation of NQO1 to counteract NO-induced apoptosis [163].

5. Conclusions

Our survey of clinical supplementation trials and studies with isolated vascular rings and cultured endothelial cells (Tables 1 and 2) highlights that soy isoflavones and polyphenols contained in red wine, green and black tea and dark chocolate rapidly activate intracellular kinase signaling pathways, resulting in eNOS activation and/or increased NO synthesis. Reports on the effects of antioxidants and polyphenols on redox sensitive gene expression in endothelial cells are limited, and published DNA microarray studies have not correlated changes in mRNA expression with altered enzyme activity or endothelial function [164].

The schematic model in Fig. 3 summarizes potential mechanisms by which polyphenols contained in soy, red wine, tea and dark chocolate elicit transactivation of vasoactive (e.g. eNOS) and antioxidant defense (e.g. HO-1, NQO1, xCT) genes. Further studies are required to establish whether soy isoflavones bind specifically to plasma membrane estrogen receptors, including the classical receptors ERα and ERβ [24], truncated estrogen receptors [122] and/or GPR30, a G-protein coupled receptor with characteristics of membrane estrogen binding activity [165]. To our knowledge there is no definitive evidence that polyphenols in red wine, green or black tea and dark chocolate interact with a membrane receptor to activate intracellular signaling pathways.
Both laminar shear stress [166,167] and polyphenols (see Table 2 and [168]) increase NO and ROS production in endothelial cells. Exposure of human endothelial cells to laminar shear stress, but not oscillatory flow, is associated with Nrf2/ARE mediated induction of NADPH oxidase, heme oxygenase-1 (HO-1), and heme oxygenase-1 (HO-1) [169,170]. Activation of Nrf2/ARE mediated transcription by laminar shear stress does not apparently involve NO, whereas inhibitors of xanthine oxidase, NADPH oxidase and the mitochondrial respiratory chain suppress shear stress in vascular and inflammatory diseases [174] by modulating key redox sensitive gene transcription via NF-κB signaling pathways. In view of the inherent genetic variability and the potential for non-compliance in human supplementation trials, studies in genetically identical mice (wildtype and knockout) should provide important insights into the mechanisms by which dietary soy isoflavones and other polyphenols regulate antioxidant gene expression in endothelial and other vascular cell types, including smooth muscle cells, monocytes and platelets.

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