

## Isoflavones and endothelial function

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Dietary isoflavones are thought to be cardioprotective due to their structural similarity to oestrogen. Oestrogen is believed to have beneficial effects on endothelial function and may be one of the mechanisms by which premenopausal women are protected against CVD. Decreased NO production and endothelial NO synthase activity, and increased endothelin-1 concentrations, impaired lipoprotein metabolism and increased circulating inflammatory factors result from oestrogen deficiency. Oestrogen acts by binding to oestrogen receptors  $\alpha$  and  $\beta$ . Isoflavones have been shown to bind with greater affinity to the latter. Oestrogen replacement therapy is no longer thought to be a safe treatment for prevention of CVD; isoflavones are a possible alternative. Limited evidence from human intervention studies suggests that isoflavones may improve endothelial function, but the available data are not conclusive. Animal studies provide stronger support for a role of isoflavones in the vasculature, with increased vasodilation and endothelial NO synthase activity demonstrated. Cellular mechanisms underlying the effects of isoflavones on endothelial cell function are not yet clear. Possible oestrogen receptor-mediated pathways include modulation of gene transcription, and also non-genomic oestrogen receptor-mediated signalling pathways. Putative non-oestrogenic pathways include inhibition of reactive oxygen species production and up regulation of the protein kinase A pathway (increasing NO bioavailability). Further research is needed to unravel effects of isoflavones on intracellular regulation of the endothelial function. Moreover, there is an urgent need for adequately powered, robustly designed human intervention studies in order to clarify the present equivocal findings.

### Isoflavones: Endothelial function: Oestrogen: Cardiovascular disease

#### Introduction

Soyabean (*glycine max*), the main dietary source of isoflavones, have been cultivated in East Asia for about 5000 years. This crop is one of the world's most important sources of protein, and can be used in many applications (foodstuffs, oils, industrial products, medicine, animal feed). Soya ingredients and traditional soya foods contain 0.3–1.6 mg isoflavones/g (Wang & Murphy, 1994). Reported dietary isoflavone intake varies markedly around the world (Fig. 1), but differences in soyabean isoflavone concentrations and processing methods between geographical areas, and variation in accuracy of food composition databases, contribute significant uncertainty to estimates. In parts of Asia, intakes of isoflavones of 30–40 mg/d are common, whereas in Western countries typical isoflavone intakes are less than 1 mg/d.

Dietary isoflavones have been suggested to be protective against a range of diseases, including osteoporosis, cancer and cardiovascular diseases, as well as alleviating menopausal symptoms in postmenopausal women. The lower rates of CVD in areas of south east Asia compared with Western countries, especially Japan, are thought to be partly attributable to the greater consumption of soya foods in these countries (for example, Beaglehole, 1990). However, this observation does not rule out the effects of other lifestyle factors, such as lower dietary intakes of saturated fat or higher intakes of fresh vegetables and/or fish. In addition, isoflavones are not the only constituent of soya that may be responsible for the protective effects.

Isoflavones (members of the class of flavonoids) belong to a group of plant compounds called phyto-oestrogens. Phyto-oestrogens are of great interest in the field of

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**Abbreviations:** ADMA, asymmetrical dimethylarginine; cAMP, cyclic adenosine 3', 5' cyclic monophosphate; CRP, C-reactive protein; eNOS, endothelial NO synthase; ER, oestrogen receptor; ET-1, endothelin-1; FBF, forearm blood flow; FMD, flow-mediated dilation; HUVEC, human umbilical vein endothelial cells; ICAM, intracellular adhesion molecule; MAPK, mitogen-activated protein kinase; MCP, monocyte chemotactic protein; PI3K, phosphatidylinositol 3'-kinase; PGI<sub>2</sub>, prostacyclin; PKA, protein kinase A; ROS, reactive oxygen species; TNF- $\alpha$ , tumour necrosis factor- $\alpha$ ; VCAM, vascular cell adhesion molecule; vWF, von Willebrand factor.

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Fig. 1. Dietary isoflavone intake in different countries.

nutrition and health due to their potential oestrogenic properties in the body. Fig. 2 shows the close structural relationship of genistein and daidzein compared with  $17\beta$ -oestradiol. Although oestrogenicity assays have reported low oestrogenic potency for dietary isoflavones (100–1000 times less than  $17\beta$ -oestradiol; Miksicek, 1993), the fact that when dietary intakes of isoflavones are high their circulating levels could exceed endogenous oestradiol concentrations by up to 10 000-fold (Adlercreutz *et al.* 1993) suggests potential physiological effects.

Soyabeans contain three main isoflavones; genistein, daidzein and glycitein, present in one of four chemical

forms: aglycones,  $\beta$ -glucosides, acetyl- $\beta$ -glucosides or malonyl- $\beta$ -glucosides. Isoflavones predominantly occur in plants as the water-soluble  $\beta$ -glucosides, genistin, daidzin, glycitin (Song *et al.* 1998), which are hydrolysed by intestinal and bacterial  $\beta$ -glucosidases. Genistein, daidzein and glycitein may be absorbed or further metabolised by gut microflora enzymes to isoflavone metabolites such as equol, dihydrogenistein, dihydrodaidzein, 6'-hydroxy-O-desmethylangolensin and -O-desmethylangolensin before absorption. Isoflavone metabolism is reviewed in more detail elsewhere (Bingham *et al.* 2003).

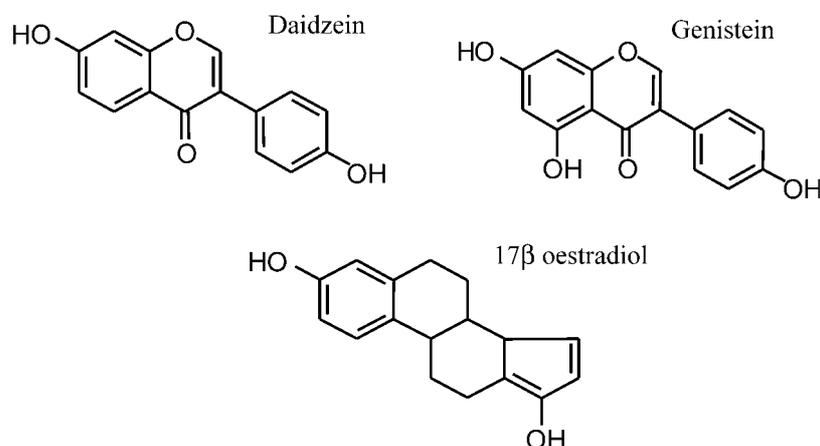


Fig. 2. The structure of genistein, daidzein and oestradiol.

There is substantial evidence that oestrogen has beneficial effects on the cardiovascular system by enhancing NO production and thereby maintaining normal endothelial vasodilatory response and integrity of the vascular system. Oestrogen-like compounds such as isoflavones are also suggested to protect the endothelium and therefore be protective against the development of CVD. The present paper will provide a brief background to current understanding of the pathophysiology leading to endothelial dysfunction, the relationship between the endothelium function and oestrogen, and evidence for a protective role for isoflavones provided by human intervention studies and mechanistic studies in animal and cell models.

### Endothelial function

#### *The vascular endothelium*

The endothelium of the vascular system is effectively a large organ with multiple functions in addition to its main role as a physical barrier separating the blood from the vessel wall. This single layer of cells is essential for the regulation of blood flow, vascular tone, vascular smooth muscle growth, inflammation, coagulation and fibrinolysis. A healthy endothelium regulates blood flow by inducing relaxation or constriction responses in the underlying smooth muscle cell of the blood vessel. Endothelium-dependent vasodilation occurs when factors released from the endothelium cause the underlying smooth muscle to relax and thereby dilate the vessel, and is mainly mediated by release of NO. NO is a gaseous molecule synthesised from arginine. It is found throughout the body, but in the endothelium it is produced by the isoform of NO synthase called endothelial NO synthase (eNOS).

NO released by the endothelial cell diffuses to the smooth muscle layer where it relaxes the smooth muscle cells. It also acts in the lumen by inhibiting adhesion of leucocytes to the endothelium and release of inflammatory cytokines. In addition, NO inhibits platelet aggregation and therefore protects against thrombosis. Other endothelium-dependent vasodilatory factors include prostacyclin (PGI<sub>2</sub>), substance P, bradykinin, and endothelium-derived hyperpolarising factor. The endothelium also secretes vasoconstricting factors, the major vasoconstrictor being the peptide endothelin-1 (ET-1), in addition to other endothelial factors such as angiotensin II, thromboxane A<sub>2</sub>, prostaglandins and H<sub>2</sub>O<sub>2</sub>. The response of the vasculature to adverse physico-chemical stimuli will depend on the relative production of vasodilatory and vasoconstrictive stimuli, as well as the permissive effects of local and systemic hormones which can modulate the intracellular response to these potent vasoactive compounds. Within this context there is clearly also potential for circulating dietary components, such as isoflavones, to influence both the release and intracellular action of vasoregulatory agents.

#### *Role of endothelial dysfunction in cardiovascular disease*

Prolonged activation of vascular mechanisms for protecting against adverse physico-chemical stimuli (inflammatory response, procoagulation and vasoconstriction) can lead to

endothelial dysfunction, an early event in the progression of atherosclerosis. Many factors have the potential to accelerate the progression of endothelial dysfunction with age. It is not surprising to note that many of these factors are the well-recognised classic risk factors for CVD, since endothelial dysfunction is a major component of the aetiology of stroke, thrombosis, atherosclerosis and heart disease. Factors that have been shown to be associated with endothelial dysfunction include: smoking, which impairs endothelium-dependent dilation by inactivating eNOS, increasing oxidative stress and increasing adhesion of monocytes to the endothelium (Zeiher *et al.* 1995); dyslipidaemia, which also leads to endothelial dysfunction through disruption of eNOS as well as pro-oxidant and inflammatory stimulation (Cai & Harrison, 2000); insulin resistance and type 2 diabetes, closely related to endothelial dysfunction (Hayden & Tyagi, 2003); hypertension, clearly directly related to endothelial dysfunction, and other factors including physical activity (Hambrecht *et al.* 2003) and plasma homocysteine levels (Chen *et al.* 2002); oestrogen deficiency, a pertinent contributing cause of endothelial dysfunction in women which will be covered in the next section.

#### *Methods for assessing in vivo endothelial function*

*Measurement of endothelium-dependent vasodilation.* The first method used to measure *in vivo* endothelial function involved an invasive and time-consuming procedure to measure coronary artery diameter and blood flow. It was demonstrated that acetylcholine infusion caused the coronary arteries of healthy subjects to dilate, whereas the opposite happened in patients with coronary artery disease (Ludmer *et al.* 1986). Acetylcholine was used as this causes the endothelium to release NO and therefore vasodilation in a healthy artery, whereas in a diseased blood vessel NO production is diminished and acetylcholine acts directly on the smooth muscle to cause vasoconstriction.

Since the relationship between CHD and endothelial function exists for peripheral vessels as well as coronary arteries (Neunteufl *et al.* 1997), some non-invasive methods were introduced for the measurement of endothelium-dependent vasodilation. One of these uses ultrasound to record images of the endothelium-dependent dilatory responses of the brachial artery caused by reactive hyperaemia (flow-mediated dilation; FMD) (Celermajer *et al.* 1992). FMD is caused by shear stress-induced generation of endothelium-dependent vasodilators, mainly thought to be NO. Another non-invasive method involves venous occlusion plethysmography to measure forearm blood flow (FBF) in peripheral resistance vessels following infusion of acetylcholine (Fichtlscherer *et al.* 2000). A third non-invasive method uses laser Doppler imaging to measure peripheral microvascular endothelial function, a technique that assesses the response of cutaneous blood vessels to transdermal delivery of endothelium-dependent (for example, acetylcholine) and endothelium-independent (for example, sodium nitroprusside) vasoactive agents by iontophoresis (Morris & Shore, 1996). These methods have been widely adopted and shown to be reasonable prognostic indicators of cardiovascular events in patients

with vascular diseases (Caballero *et al.* 1999; Heitzer *et al.* 2001; Gokce *et al.* 2003; Farkas *et al.* 2004; Hansell *et al.* 2004). Measurements of arterial compliance (the elasticity of conduit arteries) are also commonly used as an indicator of endothelial function, since the former is partly dependent on the latter (Nigam *et al.* 2003).

**Circulating biomarkers.** Circulating inflammatory factors are commonly used as surrogate markers of endothelial dysfunction; for example, cell adhesion molecules, cytokines and C-reactive protein (CRP). Since epicardial endothelium-dependent vasodilation seems to be entirely dependent on the bioavailability of NO in the endothelium (Quyyumi *et al.* 1995), then measurement of NO production would seem to be an ideal circulating predictor of endothelial function. Although there are assays available to measure NO production *in vitro*, it is not possible to measure NO *per se* in blood. However, plasma NO metabolites, NO(x) (the sum of nitrite and nitrate), have been widely used to assess NO bioavailability *in vivo*. This method has been used as a biomarker of endothelial function (Thambyrajah *et al.* 2001) and cardiovascular risk (Ishibashi *et al.* 1999). However, recent investigations have cast doubt on the validity of measuring NOx; only plasma nitrite was shown to be an accurate indicator of eNOS activity and related closely to FBF, whereas nitrate and NOx showed no reliable associations (Lauer *et al.* 2001, 2002; Kleinbongard *et al.* 2003).

ET-1, the most potent vasoconstrictor, is closely linked to NO production in the endothelial cell, and therefore may also be a useful candidate marker for endothelial function. As a circulating biomarker it is limited by the fact that it is not secreted in an endocrine fashion into the lumen of the blood vessel, but instead is released directly towards the smooth muscle cells (Wagner *et al.* 1992). However, some ET-1 is discharged into the circulation, and despite the short half-life (Weitzberg *et al.* 1993) there is ample evidence that high plasma levels are associated with cardiovascular diseases (Ommand *et al.* 1994).

An increased plasma concentration of CRP, an inflammatory molecule considered to be a strong predictive

biomarker of coronary risk, is related to reduced vasodilation in patients with coronary artery disease (Fichtlscherer *et al.* 2000), and also reduced NO bioavailability (Fichtlscherer *et al.* 2004). Von Willebrand factor (vWF), a glycoprotein secreted by the endothelial cell with important roles in platelet aggregation and adhesion, has also been suggested to be an indicator of endothelial dysfunction (Blann, 1993). The cell adhesion molecules, including intracellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM), and E-selectin, are expressed on the surface of the activated endothelial cell where they bind and attract circulating leucocytes to infiltrate the endothelium. Raised plasma concentrations have been linked with cardiovascular pre-clinical and disease states (Hwang *et al.* 1997; Wojakowski & Gminski, 2001), and also endothelial function (Brevetti *et al.* 2001). These indicators of endothelial dysfunction and other putative circulating biomarkers of endothelial function are listed in Table 1.

### Oestrogen and endothelial function

The decline in ovarian function and resulting oestrogen deficiency that occurs with menopause has significant effects on the health of middle-aged and elderly women. The average age of menopause for British women is 50 years (Lawlor *et al.* 2003) and average life expectancy at birth for women in the UK is now 80 years (Micheli *et al.* 2003). This leaves, on average, 30 years when a woman will be at a greater risk of CVD through oestrogen deficiency in addition to the effects of ageing. There is a large acceleration in the incidence of CVD among women following the menopause (Kannel & Wilson, 1995). Many studies have demonstrated the decline in cardiovascular health following ovariectomy in animal models, and reduction of cardiovascular risk biomarkers by oestrogen administration in ovariectomised animals (Wagner *et al.* 1991; Adams *et al.* 1995). Some of the increase in cardiovascular risk after the menopause is due to changes in lipoprotein metabolism, but a major contributing factor is also the impairment of endothelial function.

**Table 1.** Circulating biomarkers of endothelial function

	Circulating biomarkers	Raised or decreased with endothelial dysfunction?
Markers of dilation	Endothelin-1	+
	NO metabolites (NOx)	-
	Prostacyclin	-
	Angiotensin II	+
	Thromboxane A2	+
	Asymmetric dimethylarginine	+
	Endothelium-derived hyperpolarising factor	-
Inflammation	Cell adhesion molecules	+
	Monocyte chemotactic protein-1	+
	C-reactive protein	+
	Cytokines: IL-1, IL-6, tumour necrosis factor- $\alpha$	+
	Secretory non-pancreatic type II phospholipase A(2)	+
Haemostasis	Plasminogen activator inhibitor-1	+
	Tissue plasminogen activator	-
	Thrombomodulin	+
	Von Willebrand factor	+

+ , Raised; - , decreased.

### *Protective effects of oestrogen against endothelial dysfunction*

It is well documented that oestrogen has protective effects on the endothelium. There are many studies that illustrate the vasodilatory effects of oestrogen, mediated via activation of eNOS and thereby increased NO production. For example, in postmenopausal women, acute administration of oestradiol increased endothelium-dependent vasodilation in the coronary arteries (Gilligan *et al.* 1994), and a course of oral oestradiol increased endothelium-dependent vasodilation in the brachial artery (Lieberman *et al.* 1994). Oestrogen has also been shown to improve response to vascular injury (i.e. the thickening of the medial wall and smooth muscle cell proliferation) and re-endothelialisation (regrowth of the endothelium following removal), techniques that simulate the formation of atherosclerotic lesions in animal models (Sullivan *et al.* 1995; Krasinski *et al.* 1997).

The major effect of oestrogen on the endothelial cell is NO production.  $17\beta$ -Oestradiol increases NO release (Gisclard *et al.* 1988), and up regulates eNOS (for a review, see Chambliss & Shaul, 2002). Oestrogen-induced NO release occurs by more than one pathway, as indicated by the demonstration of both acute and chronic effects on NO production and eNOS activity. Oestrogen can act through the classical oestrogen receptor (ER)–transcriptional pathway, whereby eNOS gene expression is increased and NO production increases over hours or days. These are referred to as the ‘genomic’ effects of oestrogen. However, the acute effects, which are rapid in response and short in duration, may involve ER $\alpha$ -mediated activation of non-transcriptional pathways (non-genomic) (Darblade *et al.* 2002), or may be mediated via direct activation of cell-signalling pathways. For instance, once oestrogen has bound to the ER, the acute activation of eNOS is effected by stimulation of Ca<sup>2+</sup> release from the endoplasmic reticulum, which binds to calmodulin leading to the dissociation of eNOS from caveolin-1 in the plasma membrane caveolae, and its translocation to the cytosol. The phosphatidylinositol 3'-kinase (PI3K) cell-signalling cascade is also activated, leading to rapid activation of eNOS (Chambliss *et al.* 2000; Simoncini *et al.* 2003). Oestrogen-bound ER $\alpha$  (and even non-ligand-activated ER) can also rapidly activate eNOS via the mitogen-activated protein kinase (MAPK) and tyrosine kinase pathways (Simoncini & Genazzani, 2003). Oestrogen also appears to increase NO availability by modulating reactive oxygen species (ROS) and antioxidant status within the endothelial cell. Oestrogen has been shown to inhibit the production of NADPH oxidase and superoxide, thus attenuating the potential for superoxide-mediated degradation of NO and formation of peroxynitrites (Hernandez *et al.* 2000; Wagner *et al.* 2001; Xu *et al.* 2004b). In addition to its positive effects on NO, oestrogen has been shown to promote the release of other vasodilator agents from endothelium. PGI<sub>2</sub> production is stimulated (via the cyclooxygenase pathway) in cultured endothelial cells (Mikkola *et al.* 1996), endothelial tissue (Makila *et al.* 1982), and

postmenopausal women when treated with oestrogen (Viinikka *et al.* 1997).

Oestrogenic beneficial effects on endothelial function have also been attributed to the inhibition of vasoconstrictors such as ET-1, thromboxane A2 and angiotensin II receptor. There is a strong base of evidence to show that oestrogen reduces ET-1. *In vitro* oestrogen was shown to attenuate ET-1 production in cultured endothelial cells (Akashita *et al.* 1996; Wingrove & Stevenson, 1997) and *in vivo* oestrogen was shown to acutely reduce coronary plasma ET-1 levels in postmenopausal women with CHD (Webb *et al.* 2000), and following long-term treatment oestrogen reduced plasma ET-1 concentrations in postmenopausal women with increased cardiovascular risk factors (Wilcox *et al.* 1997). Asymmetrical dimethylarginine (ADMA) is an endogenous inhibitor of eNOS (Vallance *et al.* 1992), and a predictive factor for coronary events (Valkonen *et al.* 2001). A recent study showed that plasma levels of ADMA were reduced following 2 years of oestrogen treatment in hysterectomised postmenopausal women (Teerlink *et al.* 2003). It is not yet clear how oestrogen acts to reduce ADMA, but it has been suggested that it increases the activity of an enzyme, dimethylarginine dimethylaminohydrolase, which is responsible for degrading ADMA (Holden *et al.* 2003).

As well as direct effects on the endothelial vasodilator and constrictor pathways, oestrogen is known to reduce many of the metabolic and molecular pathways that lead to the formation of atherosclerotic lesions and thereby impact on endothelial function. Beneficial effects of oestrogen treatment include: decreased LDL-cholesterol (Floter *et al.* 2004) and increased HDL-cholesterol (Paganini-Hill *et al.* 1996); decreased expression of haemostatic and inflammatory factors (Koh *et al.* 1997a,b). However, oestrogen has less beneficial effects on triacylglycerol concentrations, with increased levels observed following oestrogen replacement therapy (Kuller, 2003).

### *Oestrogen receptors $\alpha$ and $\beta$*

There are two known ER; ER $\alpha$  (cloned in 1986 by Green *et al.* (1986) and Greene *et al.* (1986)) and ER $\beta$  (Kuiper *et al.* 1996), both widely distributed throughout the body. Oestrogen, or other oestrogenic ligands, bind to these nuclear receptors in the cytosol, which allows the receptor to enter the nucleus and bind to response elements on DNA, and modulate gene transcription. ER $\alpha$  and ER $\beta$  bind to the same oestrogen response elements (therefore affecting transcription of the same genes); however, they are functionally distinct and will not necessarily affect these genes in the same direction (Paech *et al.* 1997) or with the same potency (Weatherman & Scanlan, 2001). Although ER $\alpha$  and ER $\beta$  are both expressed throughout the vasculature (Register & Adams, 1998), ER $\beta$  is the receptor that predominates in this tissue, particularly in women (Hodges *et al.* 2000). In addition, there are specific functional domains on the two receptor proteins that have a degree of divergence in their homology, which may affect binding of oestrogenic ligands (Kuiper *et al.* 1996; Mosselman *et al.* 1996); this is illustrated by the greater binding affinity of

isoflavones for ER $\beta$  compared with ER $\alpha$  (Morito *et al.* 2001).

Since ER $\alpha$  and ER $\beta$  have different tissue distributions and binding affinities, it seems probable that the protective effects of oestrogen, or indeed oestrogen-like compounds, on the endothelium would vary with receptor type. Studies using knockout mice (ER $\alpha$ KO, ER $\beta$ KO and ER $\alpha,\beta$ KO) showed that ER $\alpha$  was the mediator of the inhibitory effects of oestrogen on the vascular injury response (Karas *et al.* 1999, 2001, Pare *et al.* 2002) and the improved endothelial regrowth following de-endothelialisation, that occurred with oestrogen treatment (Brouchet *et al.* 2001).

These results appear to indicate that ER $\alpha$  is the most important receptor for the protective effects of oestrogen in the endothelium. However, studies from two independent groups found that ER $\beta$  mRNA expression increases to high concentrations after arterial vascular injury (Lindner *et al.* 1998; Aavik *et al.* 2001), whereas ER $\alpha$  mRNA was expressed only at low background levels. Therefore it is puzzling that ER $\beta$ KO mice retain their response to oestrogen following vascular injury (Karas *et al.* 1999). ER $\alpha$  and ER $\beta$  bind to the oestrogen response element of target genes as homo- and heterodimers. Studies have shown that ER $\alpha$  and ER $\beta$  heterodimers are more likely to be formed compared with homodimers of ER $\beta$  and that the latter activate transcription to a lesser extent than ER $\alpha$  homodimers (Cowley *et al.* 1997). Therefore, an absence of ER $\alpha$  may affect response to oestrogen in the artery to a greater extent than an absence of ER $\beta$ .

Since isoflavones have a greater binding affinity for ER $\beta$  compared with ER $\alpha$  then it might be expected that any protective effect of isoflavones against vascular injury would be weak compared with that of oestrogen, unless the isoflavone–ER $\beta$  conformation induced a greater affinity for the oestrogen response element than the oestrogen–ER $\beta$  conformation (Kostelac *et al.* 2003). Experiments conducted by An *et al.* (2001) showed that not only do isoflavones bind more effectively to ER $\beta$  compared with ER $\alpha$ , but they are also 1000 times more potent at generating transcriptional activity via ER $\beta$  compared with ER $\alpha$ , due to selective recruitment of co-regulators to ER $\beta$ . In addition, isoflavones are more effective at triggering transcriptional repression rather than activation, suggesting that isoflavones may repress transcription of some genes that are normally activated by ER $\alpha$ . Further studies are clearly required to untangle the relative roles of the ER subtypes in oestrogen- and oestrogen-like ligand-mediated vascular protection.

#### *Hormone and oestrogen replacement therapy*

Due to beneficial effects on LDL-cholesterol, hormone replacement therapy was widely advocated as an effective means of delaying the progression of atherosclerosis in postmenopausal women. In fact there is substantial evidence that hormone replacement therapy treatment, mainly oestrogen alone rather than combined oestrogen + progesterone, improves vasodilation (for example, Lieberman *et al.* 1994; McCrohon *et al.* 1996; Bush *et al.* 1998; Koh *et al.* 2001). However, lack of efficacy of hormone replacement therapy with respect to CHD progression, clear evidence of increased risk of thrombosis (Nelson *et al.*

2002) and increased levels of the inflammatory marker, CRP (Ridker *et al.* 1999), as well as hormone-dependent cancers (Beral & Million Women Study Collaborators, 2003), has led to a search for alternative therapies.

#### **Isoflavones and endothelial function**

In assessing the evidence for a role of isoflavones in endothelial function, the present review will incorporate *in vivo* studies that have investigated endothelium-dependent vasodilation, those that have measured NO production and/or eNOS activity, and those that have measured circulating biomarkers of endothelial function (such as those that regulate monocyte adhesion and vascular constriction). The aim of the present review is to consider the evidence for potential beneficial effects of isoflavones independent of other components in foods in which they are found. For this reason, only *in vivo* studies that have used isolated isoflavones are included, since isoflavones administered via soya protein or soya foods have been shown to have different effects from isoflavones alone, for example, their effects on blood lipids. Mechanisms for isoflavone-mediated effects on endothelial function will be explored, using evidence from animal and cell culture experiments.

#### *Human studies*

Habitual dietary isoflavone intakes have been correlated with markers of endothelial function. A negative association between dietary isoflavone intake and aortic stiffness was shown in 403 postmenopausal Dutch women (van der Schouw *et al.* 2002), despite the low intakes typical of a Western diet. However, another cross-sectional study could not demonstrate the same relationship (Kreijkamp-Kaspers *et al.* 2004). Acute studies in human subjects have shown that arterial infusion of genistein, but not daidzein, greatly increased FBF, similar to that observed with 17 $\beta$ -oestradiol (Walker *et al.* 2001). In addition, increased FBF was shown following arterial infusion of the isoflavone metabolite, dehydroequol (Chin-Dusting *et al.* 2004), suggesting that the gut metabolism of dietary isoflavones may be important for their systemic effects.

The number of human intervention studies that have investigated the effects of isolated-isoflavone supplementation on endothelial function is low. Table 2 shows a summary of controlled human intervention studies using isoflavone supplementation to investigate endothelial function. Some studies have found positive effects on *in vivo* endothelial function measurements (Nestel *et al.* 1997, 1999; Squadrito *et al.* 2002, 2003), although the latter two studies appear to have reported on the same study group. Others, however, found no effect of isoflavones on *in vivo* endothelial function (Simons *et al.* 2000; Hale *et al.* 2002; Lissin *et al.* 2004). A few studies have investigated effects on different circulating biomarkers of endothelial function, with negative findings for fibrinogen, CRP, E-selectin and NOx (Nikander *et al.* 2003; Teede *et al.* 2003; Atkinson *et al.* 2004), but beneficial effects reported for isoflavones on plasminogen activator inhibitor-1 (perimenopausal women only), ET-1, VCAM-1 and NOx (Squadrito *et al.* 2002, 2003; Teede *et al.* 2003; Atkinson *et al.* 2004). Many of

**Table 2.** Human intervention trials: effects of isoflavones on *in vivo* endothelial function and putative biomarkers of endothelial function

Reference	Subject characteristics	Study design	Duration of study	Food or supplement description	Aglycone dose and composition	Measure	Outcome
Atkinson <i>et al.</i> (2004)	177 pre-, peri- and postmenopausal W	R, SB, P, Pa	12 months	Red clover isoflavone (43.5 mg/d) tablets	Biochanin A (26 mg/d), formononetin (16 mg/d), genistein (1 mg/d), daidzein (0.5 mg/d)	Fibrinogen PAI-1	NC ↓ (Perimenopausal only)
Hale <i>et al.</i> (2002)	29 PMW, > 1 year since last menstruation, USA	R, DB, P, Pa	2 weeks	Soya isoflavone (80 mg/d) tablets v. placebo	Not reported	FMD	NC
Lissin <i>et al.</i> (2004)	40 PMW, USA	R, DB, P, Pa	6 weeks	Soya isoflavone (90 mg/d) tablets	Genistein (44.6 mg/d), daidzein (44.6 mg/d), glycitein (0.8 mg/d)	FMD Endothelium-independent vasodilation	NC ↑
Nestel <i>et al.</i> (1997)	28 Perimenopausal women and PMW, Australia	R, SB, P, CO	15 weeks per arm	Soya isoflavone (80 mg/d) tablets v. placebo	Genistein (45 mg/d), daidzein (33 mg/d), glycitein (2 mg/d)	Arterial compliance	↑
Nestel <i>et al.</i> (1999)	14 PMW, > 1 year since last menstruation, Australia	R, DB, P, sequential treatments	15 weeks (5 week treatment)	Red clover isoflavone (40 mg/d or 80 mg/d) tablets v. placebo	40 mg Tablet: genistein (4 mg), daidzein (3.5 mg), biochanin A (24.5 mg), formononetin (8 mg)	Arterial compliance	↑
Nikander <i>et al.</i> (2003)	56 PMW, History of breast cancer, Finland	R, DB, P, CO	3 months per arm	Isoflavone (114 mg/d) tablets	Glycitein (66 mg/d), daidzein (42 mg/d), genistein (6 mg/d)	CRP E-selectin NOx	NC NC NC
Simons <i>et al.</i> (2000)	20 PMW, > 1 year since last menstruation, Australia	R, DB, P, CO	8 weeks per arm	Soya isoflavone tablets (80 mg/d) v. placebo		FMD	NC
Squadrito <i>et al.</i> (2002)	60 PMW, > 1 year since last menstruation, Italy	R, DB, P, Pa	6 months	Genistein (54 mg/d) tablets (n 30) v. placebo	Genistein (54 mg/d)	NOx Endothelin-1 FMD	↑ ↓ ↑
Squadrito <i>et al.</i> (2003)	79 PMW, > 1 year since last menstruation, Italy	R, DB, P, Pa	12 months	Genistein (54 mg/d) tablets (n 27) v. HRT or placebo	54 mg/d genistein	NOx Endothelin-1 FMD	↑ ↓ ↑
Teede <i>et al.</i> (2003)	80 M and W, Australia	R, DB, P, CO	6 weeks per arm	Red clover isoflavone (80 mg/d) tablets v. placebo	Biochanin (n 40) or formononetin (n 40) (80 mg/d) v. placebo	Arterial stiffness VCAM-1 FMD	↓ (Formononetin) ↓ (Formononetin) NC

W, women; R, randomised; SB, single-blind; P, placebo-controlled; Pa, parallel; PAI-1, plasminogen activator inhibitor-1; NC, no change; ↓, decreased; PMW, postmenopausal women; DB, double-blind; FMD, flow-mediated dilatation; ↑, increased; CO, cross-over design; CRP, C-reactive peptide; NOx, NO metabolites; HRT, hormone replacement therapy; M, men; VCAM-1, vascular cell adhesion molecule-1.

these human intervention studies have used small subject numbers and therefore it may be unwise to attach great significance to their findings. In addition, in a large group of postmenopausal European women ( $n$  117), a recent randomised cross-over placebo-controlled dietary intervention trial found 50 mg isoflavones/d for 8 weeks to have no effect on plasma CRP, VCAM-1, ICAM-1, E-selectin, monocyte chemoattractant protein (MCP)-1, ET-1 or vWF (WL Hall, K Vafeiadou, J Hallund, S Bugel, C Koenig, M Reimann, M Ferrari, F Branco, D Talbot, J Powell, T Dadd and CM Williams, Unpublished results).

There is some evidence from these studies to suggest a beneficial effect of isoflavones on *in vivo* measures of endothelial function such as FMD and arterial compliance, but there are also studies that contradict these findings. Three trials found possible beneficial effects on *in vivo* endothelial function, originating from two laboratories. Four other studies show no effect on FMD. A single group (Squadrito *et al.* 2002, 2003) has found reduced ET-1 levels and increased markers of NO production, but it is not clear whether these studies represent two separate groups of women. These results need to be replicated by other groups, especially in the light of recent opinion regarding the validity of using nitrites + nitrates as an indicator of NO production (Lauer *et al.* 2002; Kleinbongard *et al.* 2003). Since the biomarkers used differ between studies, it is not yet possible to draw conclusions regarding effects of isoflavones on circulating biomarkers of endothelial function. Overall, whilst these studies provide some promising indicators, there is need for replication of the positive findings, in other laboratories and using a wider range of *in vivo* measures and circulating biomarkers.

#### *Animal studies: mechanisms*

Whilst the data from human studies remain sparse and conflicting, animal studies appear to provide a clearer indication of benefit. There is persuasive evidence from animal studies that isoflavones can improve endothelial function. Genistein and daidzein were shown to produce vasodilatory effects when incubated with arterial rings or aortic strips, although this appeared to be endothelium-independent (Nevala *et al.* 1998; Li *et al.* 2004). However, genistein, daidzein and isoflavone metabolites increased endothelium-dependent relaxation of arterial rings (Chin-Dusting *et al.* 2001; Karamsetty *et al.* 2001) and abdominal aorta (Jiang *et al.* 2003). *In vivo* animal studies showed that subcutaneous infusion of isoflavones for 4 weeks increased eNOS activity and reversed endothelial dysfunction in ovariectomised rats (Squadrito *et al.* 2000). In addition, subcutaneous injection of daidzein increased NO production and arterial vasodilation, decreased caveolin-1 and increased calmodulin expression in male rats (Sobey *et al.* 2004). Interestingly, there was no change in eNOS expression, suggesting augmented eNOS activation and modulation of regulatory proteins, rather than increased eNOS transcription. Furthermore, dietary isoflavone consumption increased NOS activity and increased vasodilation in ovariectomised rats (Yamaguchi *et al.* 2001; Catania *et al.* 2002). It has also been shown that genistein treatment

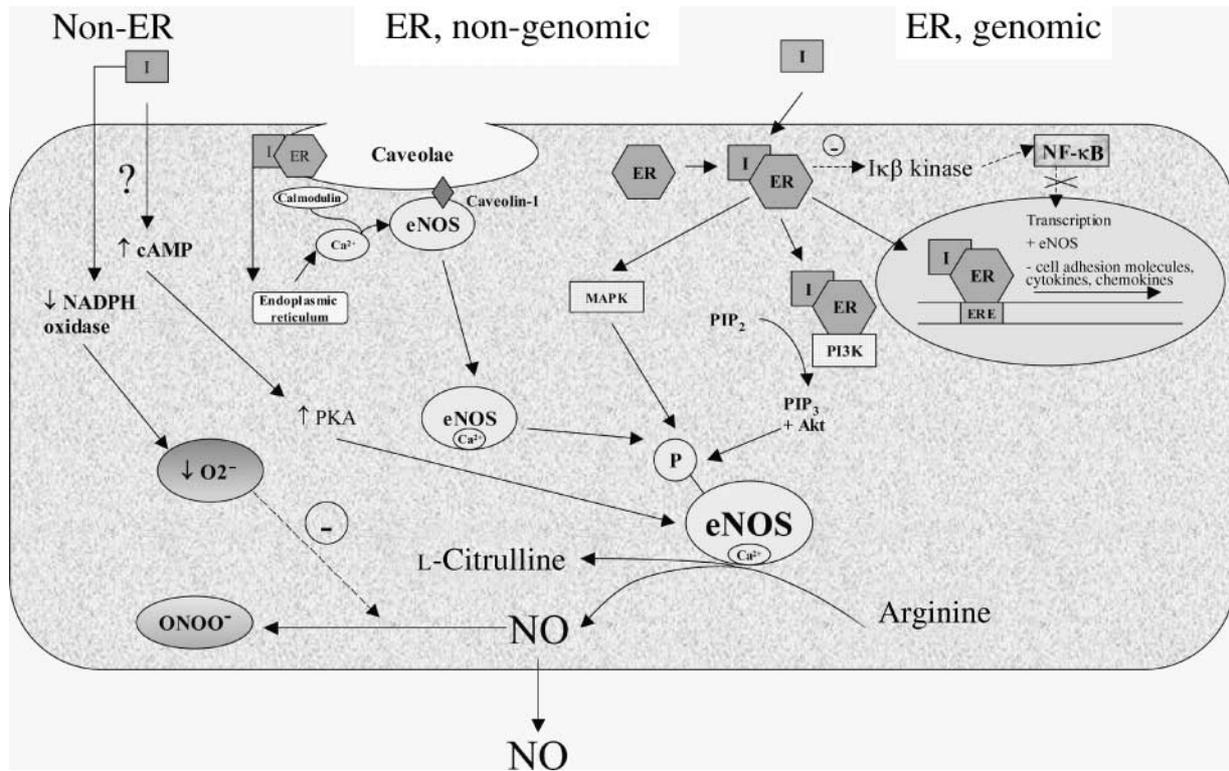
inhibits expression of inflammatory genes in ovariectomised mice, such as VCAM-1 and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) (Evans *et al.* 2001), and that daidzein enhances vasodilation by a non-NO mechanism likely to be endothelium-derived hyperpolarising factor (Woodman & Boujaoude, 2004).

#### *In vitro studies: mechanisms*

There are many cellular mechanisms whereby isoflavones may potentially improve endothelial function, and *in vitro* experiments, such as those using cultured endothelial cells, are the ideal way to understand the complexities behind these effects. Frequently, these mechanisms have been suggested based upon knowledge of the action of oestrogens on the endothelial cell. However, this may be a misleading approach with respect to the isoflavones since there is also evidence that isoflavones may improve endothelial function independently of the ER. The following section will cover some of the direct pathways of stimulation or inhibition of NO-producing or other pathways, and indirect mechanisms such as anti-inflammatory and antioxidant action.

*Nitric oxide, endothelin-1 and prostacyclin.* The mechanism for the isoflavone-induced vasodilation observed in the studies conducted to date, as with oestrogen, is assumed to be mainly through stimulation of NO production via genomic and non-genomic ER-mediated activation of eNOS. It is also possible that isoflavones act directly (i.e. not via the ER) to increase eNOS activity. Possible mechanisms for non-genomic ER-mediated isoflavone actions on the endothelial cell include modulation of phosphorylating cell pathways such as PI3K–Akt, ERK–MAPK, and protein tyrosine kinases; also by influencing Ca channels (Ji *et al.* 2002), thereby increasing intracellular Ca and enabling eNOS to dissociate from caveolin-1. Fig. 3 shows an overall diagram of various mechanisms by which isoflavones may act on the endothelial cell to increase NO release. Genistein, but not daidzein, is known to be a tyrosine kinase inhibitor (Nakashima *et al.* 1991); consequently it has been hypothesised that the vasodilatory effects may be acting through this pathway (Lee & Man, 2003). However, non-physiological genistein concentrations are needed to inhibit tyrosine kinase activity, and other isoflavones can potentiate NO production (Sobey *et al.* 2004), suggesting that pathways other than tyrosine kinase inhibition are involved.

Liu *et al.* (2004a), in an elegant series of experiments using bovine aortic endothelial cells, explored the various possible mechanisms whereby acute physiological concentrations of genistein could induce eNOS activation. These authors demonstrated that inhibition of the PI3K–Akt and ERK–MAPK pathways did not affect genistein-induced eNOS activation. An ER-antagonist also failed to abolish these effects, despite inhibiting the acute response to oestradiol. In addition, the action of genistein as a tyrosine kinase inhibitor was excluded as the likely mechanism, since daidzein had weak but significant effects on eNOS activity, plus 10 000-fold greater threshold concentrations of genistein were required to inhibit tyrosine kinase activity in



**Fig. 3.** Putative mechanisms whereby isoflavones may increase nitric production or increase NO bioavailability in the endothelial cell. I, isoflavones; ER, oestrogen receptor; ERE, oestrogen response element; PI3K, phosphatidylinositol 3'-kinase; PIP<sub>2</sub>, phosphatidylinositol-4,5-bisphosphate; PIP<sub>3</sub>, phosphatidylinositol 3,4,5-trisphosphate; eNOS, endothelial NO synthase; P, phosphate; MAPK, mitogen-activated protein kinase; cAMP, cyclic adenosine 3', 5' cyclic monophosphate; PKA, protein kinase A; O<sub>2</sub><sup>-</sup>, superoxide; ONOO<sup>-</sup>, peroxynitrite; IκB, inhibitory κB.

these cells. Finally, it was demonstrated that a protein kinase A (PKA) inhibitor completely abolished the genistein-induced eNOS activation. In addition, the PKA inhibitor did not inhibit eNOS activation by oestradiol treatment, confirming the specific nature of this response. Furthermore, these results from bovine aortic endothelial cells were confirmed in human umbilical vein endothelial cells (HUVEC; Liu *et al.* 2004a). Genistein was also shown to stimulate cyclic adenosine 3', 5' cyclic monophosphate (cAMP) production in the same experiments, confirming previous work with genistein showing the involvement of cAMP-dependent mechanisms in rat aortic rings (Satake & Shibata, 1999). Since PKA is cAMP-dependent, this suggests that genistein activates eNOS by stimulation of cAMP production, leading to PKA-mediated phosphorylation of eNOS. These interesting findings suggest an acute isoflavone-specific pathway by which these oestrogen-like components may activate eNOS, but need to be replicated by other groups, both *in vitro*, but more importantly *in vivo*.

Since there is thought to be a significant amount of interplay between ET-1 and NO production it would be expected that many studies have also shown inhibitory effects of isoflavones on ET-1. However, studies on the effects of isoflavones on ET-1 secretion are lacking. Non-physiological (1 mg/l) concentrations of genistein were shown to inhibit basal and cytokine-stimulated ET-1 production (Saijonmaa *et al.* 1998), but a great deal of work needs to be done in this area. PGI<sub>2</sub> expression is

inhibited by supraphysiological concentrations of isoflavones in HUVEC (Wheeler-Jones *et al.* 1996). However, serum from postmenopausal women who had taken isoflavone supplements for 3 and 6 months induced a greater production of PGI<sub>2</sub> when incubated with HUVEC (Garcia-Martinez *et al.* 2003). Further clues to the molecular mechanisms mediating the action of isoflavones on endothelial cells come from a recent gene expression study, which found that genistein treatment in HUVEC modulated the expression of several key genes encoding for proteins involved in vascular tone, including endothelin-converting enzyme 1, endothelin-2, oestrogen-related receptor-α and atrial natriuretic peptide receptor A precursor (Ambra *et al.* 2005).

**Inflammatory makers.** Isoflavones have also been the subject of research into their anti-inflammatory action. The activated endothelium releases cytokines such as IL-6 and TNF-α which induce expression of cell adhesion molecules and MCP-1 at the surface of the endothelial cell, mediated by the nuclear transcription factor, NF-κB. Therefore, it is of great interest that genistein attenuated NF-κB DNA binding and TNF-α release in human monocytes (Shames *et al.* 1999). Genistein, but not daidzein, inhibited TNF-α-induced NF-κB activation in cultured human lymphocytes and in *ex vivo* human lymphocytes following the consumption of 100 mg isoflavones/d for 3 weeks (Davis *et al.* 2001). Possible mechanisms for this isoflavone-mediated inhibition

of NF- $\kappa$ B activation include reduction of intracellular ROS (which phosphorylate inhibitory  $\kappa$ B thus enabling NF- $\kappa$ B to enter the nucleus), inhibition of inhibitory  $\kappa$ B kinases, or reduction of NF- $\kappa$ B translocation and DNA binding (Cassidy *et al.* 2003).

Genistein has been shown to inhibit cell adhesion molecule surface expression and monocyte cell adhesion (McGregor *et al.* 1994; Weber *et al.* 1995; May *et al.* 1996). Further to this, genistein inhibits TNF- $\alpha$ -induced VCAM-1 and ICAM-1 production from HUVEC, and both genistein and daidzein decrease MCP-1 secretion (Gottstein *et al.* 2003; Rimbach *et al.* 2004) in cultured endothelial cells. Low concentrations of genistein (1 and 10 nM) attenuated TNF- $\alpha$ -induced VCAM-1 mRNA expression in HUVEC, an effect that was reduced by an ER antagonist (Mukherjee *et al.* 2003). The fact that this occurred at low concentrations of genistein indicates that it does not inhibit NF- $\kappa$ B activity through its properties as a tyrosine kinase inhibitor. In summary, it appears that isoflavones have the potential to attenuate inflammatory responses, and this can be achieved *in vitro* at physiologically relevant isoflavone concentrations.

**Antioxidant capacity.** Although it has proven difficult to demonstrate the antioxidant potential of isoflavones in human studies (Hodgson *et al.* 1999; Samman *et al.* 1999), there is a body of evidence to show that these effects can be observed *in vitro*. The relevance of this to endothelial function is that generation of ROS in endothelial cells causes rapid degradation of NO. *In vitro* studies have indicated that equol and genistein are more potent antioxidants than daidzein, genistin, biochanin A and formononetin, with equol being more potent than genistein (for a review, see Bingham *et al.* 2003).

The cellular processes responsible for the antioxidant activity of isoflavones are not clear. Scavenging of free radicals is a potential mechanism. However, a number of isoflavones showed no significant scavenging effects on a range of radicals in a recent study (Guo *et al.* 2002). Other possible mechanisms include the inhibition of H<sub>2</sub>O<sub>2</sub> production (a source of the destructive hydroxyl radical) and stimulation of antioxidant enzymes such as catalase (Wei *et al.* 1995). An increase in endothelial cell glutathione concentrations was also observed following exposure of the cells to physiologically achievable concentrations of genistein and daidzein, which would increase the antioxidative effects of these isoflavones (Guo *et al.* 2002). Kerry & Abbey (1998) noted that genistein inhibited LDL oxidation, but that the hydrophilic isoflavone was poorly incorporated into the LDL particle. It was consequently shown (Meng *et al.* 1999) that esterification of isoflavones increased incorporation into LDL with a number of isoflavone-fatty acid esters inhibiting LDL oxidation. Most *in vitro* studies have investigated the effects of the aglycones, genistein and daidzein, for which there is a relatively low tissue exposure. However, one study showed that mono- and disulfated genistein metabolites are less effective antioxidants than their parent aglycone (Rimbach *et al.* 2004). Moreover, equol has been shown to inhibit NADPH oxidase assembly (thus inhibiting production of superoxide, resulting in reduced NO degradation) (Hwang *et al.* 2003).

Although the evidence from cell culture experiments is more abundant than that found in human studies, considerable caution should be exercised in extrapolating these findings to human health effects. Many studies have used non-physiological concentrations of isoflavones, and it has frequently been shown that nutritional-pharmacological treatments can have biphasic effects rather than a dose-response effect when a large range of physiological to supraphysiological concentrations are applied. Endothelial cells in culture are unlikely to behave as they would *in vivo* as they are in an artificial environment, without exposure to the circulating molecules in the circulation. Cells grown *in vitro* are subjected to the trauma of passaging, which has been shown to have a strong influence on ET-1 and PGI<sub>2</sub> production (Ranta *et al.* 1997), and are not exposed to normal shear forces. Furthermore, the particular endothelial cell type used for studies has a very important bearing on the outcome of isoflavone treatment, since large variation has been found in the degree to which immortalised cells secrete cell adhesion molecules, vWF, and cytokines (Lidington *et al.* 1999; Unger *et al.* 2002). Therefore, the cell strain, cell culture conditions and concentrations of isoflavone treatments should all be taken into consideration when drawing conclusions.

## Conclusion

Studies carried out to date provide some tantalising clues as to the possibility of a beneficial relationship between isoflavones and endothelial function. Isoflavones were originally put forward as having possible lipid-lowering effects in the light of the large number of studies that demonstrated the hypolipidaemic benefits of soya. It is now clear that isoflavone consumption confers no benefits to lipoprotein metabolism, as summarised in a recent meta-analysis (Yeung & Yu, 2003). However, isoflavone-induced effects on the endothelium may prove to be of greater physiological importance in CVD than the effects on lipoprotein metabolism. Preliminary results suggest that isoflavones increase vasodilation in human subjects, but evidence for beneficial effects on circulating biomarkers of endothelial function is sparse and inconclusive. Animal studies are supportive of an effect of isoflavones on the endothelium, but these cannot be used as the basis for recommending their use in human subjects and more human studies are urgently required. The relatively large body of work that has been carried out *in vitro* has demonstrated a number of plausible mechanisms whereby isoflavones may modulate endothelial cell function. These studies provide valuable information in understanding the cellular mechanisms involved in the putative protective effects of isoflavones in the vasculature, but need to be placed in context with respect to their physiological relevance.

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