

NUTRITIONAL FLAVONOIDS IMPACT ON NUCLEAR AND EXTRANUCLEAR ESTROGEN RECEPTOR ACTIVITIES

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ABSTRACT: *Flavonoids are a large group of non-nutrient compounds naturally produced from plants as part of their defence mechanisms against stresses of different origins. They emerged from being considered an agricultural oddity only after it was observed that these compounds possess a potential protective function against several human degenerative diseases. This has led to recommending the consumption of food containing high concentrations of flavonoids, which at present, especially as soy isoflavones, are even available as over-the-counter nutraceuticals. The increased use of flavonoids has occurred even though their mechanisms are not completely understood, in particular those involving the flavonoid impact on estrogen signals. In fact, most of the human health protective effects of flavonoids are described either as estrogen-mimetic, or as anti-estrogenic, while others do not involve estrogen signaling at all. Thus, the same molecule is reported as an endocrine disruptor, an estrogen mimetic or as an antioxidant without estrogenic effects. This is due in part to the complexity of the estrogen mechanism, which is conducted by different pathways and involves two different receptor isoforms. These pathways can be modulated by flavonoids and should be considered for a reliable evaluation of flavonoid, both estrogenicity and anti-estrogenicity, and for a correct prediction of their effects on human health.*

KEY WORDS: 17 β -Estradiol, Estrogen Receptor- α , Estrogen Receptor- β , Flavonoids, Gene Transcription, Signal Transduction Cascade

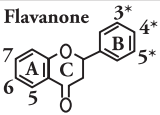
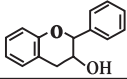
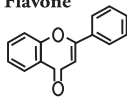
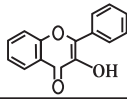
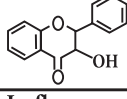
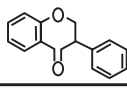
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INTRODUCTION

Some plant foods contain compounds that may have long-term effects on human and animal health besides being a source of compounds necessary for human nutrition. Among the most

important are the plant secondary metabolites flavonoids. They are present in all terrestrial vascular plants whereas, in mammals, they occur only through dietary intake (Birt et al., 2001; Dixon, 2004). More than 4,000 different flavonoids have been described and categorized into 6 subclasses as a function of the type of heterocycle involved: flavonols, flavones, flavanols, flavanonols, flavanones, and isoflavones (Table 1) (Birt et al., 2001; Manach et al., 2004).

TABLE 1: Chemical structures of certain commonly occurring plant flavonoid aglycones

Structural Formula	Representative flavonoids	Substitutions					
		5	6	7	3*	4*	5*
	Hesperitin	OH	H	OH	OH	OMe	H
	Naringenin	OH	H	OH	H	OH	H
	Catechin	OH	H	OH	OH	OH	H
	Apigenin	OH	H	OH	H	OH	H
	Luteolin	OH	H	OH	OH	OH	H
	Quercetin	OH	H	OH	OH	OH	H
	Taxifolin	OH	H	OH	OH	OH	H
	Daidzein	H	H	OH	H	OH	H
	Genistein	OH	H	OH	H	OH	H

Flavonoids, and especially isoflavones, have a long history in science. Often referred to as weak estrogens, they were chemically synthesized before the ring structure of the mammalian steroids was determined in the 1920's and 1930's (Barnes, 2004). They re-emerged from obscurity in the 1940's as the anti-estrogenic principle in red clover that caused infertility in sheep in Western Australia (Bennetts et al., 1946). This adverse effect of flavonoids, caused by interfering in some way with sex hormone actions, placed these substances in the class of endocrine-disrupting chemicals (Jacobs and Lewis, 2002). The role played by flavonoids as endocrine-disrupting chemicals have more recently been confirmed *in vivo*. Numerous effects in both male and female rats exposed to genistein from gestational day 7 into adulthood through placental transfer, lactational exposure and ingestion were observed including hyperplasia of mammary glands in both sexes, aberrant or delayed spermatogenesis, histological changes in the vagina and ovary, mineralization of renal tubules in males, modulation of natural killer cell activity, myelotoxicity, neuroendocrine changes associated with behavioural outcomes, and sexually dimorphic brain development (Flynn et al., 2000; Delclos et al., 2001; Guo et al., 2005; Doerge et al., 2006).

For the past 10–15 years scientific evidence has indicated that various forms of counter-defense have evolved in herbivores and omnivores (at least in the adult individuals) such that adult human diets rich in flavonoids lead to significantly decreased serum concentrations of total cholesterol, low-density lipoproteins (LDL) and triglycerides (Kirk et al., 1998; Ricketts et al., 2005), as well as a reduced incidence of cardiovascular diseases (Hertog et al., 1997; Cassidy et al., 2000), and osteoporosis (Dang and Lowik, 2005). These effects are recognized as estrogen-mimetic effects of dietary compounds and so are currently being explored to prevent osteoporosis (Mikkola et al., 2002; Dang and Lowik, 2005), the risk of coronary artery disease (Middleton et al., 2000; Kris-Etherton et al., 2002), and the vasomotor flushing related to estrogen deficiency in women during menopause (Mikkola et al., 2002; Fitzpatrick, 2003).

The need to develop new estrogen-mimicking agents derives from the necessity of producing their desired beneficial effects without the accompanying adverse side effects of estrogen treatment (Fitzpatrick, 2003). In fact, estrogen is a tumor-promoting agent known to increase the risk of breast and uterine cancer in women taking estrogen replacement therapy (Castagnetta et al., 2004; Yager and Davidson, 2006). On the contrary, Asian women (large isoflavonoid consumers) and vegetarians have a lower than average breast cancer risk (Limer and Speirs, 2004). In addition, flavonoids have been shown to induce responses consistent with the protective effects of fruit and vegetable rich diets against cancer in both *in vitro* test systems and small animal models (Hollman et al., 1996; Gamet-Payrastrre et al., 1999; Birt et al., 2001; Brownson et al., 2002; Keinan-Boker et al., 2004). The anticancer effect of nutritional flavonoids could represent other anti-estrogenic effects ascribed to these compounds.

As a result of all these potentially beneficial effects, flavonoids, especially soy isoflavones, have increasingly gained widespread

acceptance as safe and beneficial dietary components and they are now also available as over-the-counter nutraceuticals. The increased use of flavonoids occurs even though their mechanisms of action is not well understood. In particular, the estrogen-like or estrogen antagonistic activities of flavonoids should be better defined before recommending an increased consumption of vegetables or an alternative therapy. However, it is a fact that the molecular basis of flavonoid estrogenicity is particularly difficult to elucidate, principally due to the estrogen (E2) mechanism of action, which occurs via multiple pathways. Firstly, there are at least two estrogen receptor (ER) isoforms (*i.e.*, ER α and ER β) (Green et al., 1986; Kuiper et al., 1996), which differ in their affinity for the ligands and in their signal transduction mechanisms. Secondly, the current knowledge of E2 molecular action includes the ability of the E2-ER(s) complex both to induce gene transcription together with specific coregulators (*i.e.*, coactivators and corepressors) (Smith and O'Malley, 2004) and to evoke the membrane starting activation of specific rapid phosphorylation cascades (e.g., Src/ERK/MAPK) (Levin, 2005; Marino et al., 2005; Song et al., 2005). Both processes integrate at different levels and influence the cellular effects of estrogens. One or all of these pathways could be modulated by flavonoids, which all elicit different effects in cell/tissue. Thus, a reliable evaluation of flavonoid (anti) estrogenicity and a correct prediction of their effects on human health should take into account all estrogen-induced mechanisms. This review attempts to address this issue from recent data in order to define both what is known about flavonoid (anti) estrogenicity and where the gaps in our knowledge of this subject are.

DIETARY SOURCES, BIOAVAILABILITY, AND METABOLISM OF FLAVONOIDS

To establish evidence for the estrogenic effects of polyphenols, it is first essential to determine the nature and distribution of these compounds in the diet. Fruit and beverages such as tea and red wine constitute the main sources of polyphenols (Birt et al., 2001; Manach et al., 2004). Certain polyphenols such as quercetin are found in all plants and derivatives (fruit, vegetables, cereals, leguminous plants, fruit juices, tea, wine, infusions, etc), whereas others are specific to particular foods (e.g., flavanones in citrus fruit, isoflavones in soy, and phloridzin in apples) (Manach et al., 2004). In most cases, foods contain complex mixtures of polyphenols, which are often poorly characterized. Several factors affect the polyphenol content of plants, including ripeness at the time of harvest, environmental factors, processing, and storage (Manach et al., 2004). In 1976, Kuhnau estimated that dietary flavonoid intake in the United States was ~1 g/day and consisted grossly of the following: 16% flavonols, flavones, and flavanones; 17% anthocyanins; 20% catechins; and 45% "biflavones." Afterward, different studies provided more precise individual data concerning the intake of various classes of polyphenols. Consumption of flavonols has been estimated at ~20–25 mg/day in the United States, Denmark, and Netherlands (Sampson et al., 2002; Justesen et al., 1997; Hertog et al., 1993). In Italy,

consumption ranged from 5 to 125 mg/day, and the mean value was 35 mg/day (Pietta, 2000). The intake of flavanones is similar to or possibly higher than that of flavonols, with a mean consumption of 28.3 mg hesperetin/day in Finland (Kumpulainen, 2001). Because citrus fruit is practically the sole source of flavanones, ingestion of these substances is probably greater in regions where these fruits are produced, such as southern Europe (Manach et al., 2004). Anthocyanin consumption was studied only in Finland, where high amounts of berries are eaten, and was found to be 82 mg/day on average (Heinonen, 2001). Consumption of soy in the Asian countries is equivalent to a mean intake of 25–40 mg isoflavones/day (Coward et al., 1993). Americans and Europeans, who eat little soy, consume less than 1 milligram of isoflavones per day (de Kleijn et al., 2001). Women undergoing phytoestrogen replacement therapy for menopause consume between 30 and 70 mg isoflavones/day in the form of soy extract capsules (Manach et al., 2004).

The absorption of isoflavones by the gastrointestinal mucosa seems to partly depend on their relative hydrophobicity/hydrophilicity. There is no evidence for facilitated or active transport of flavonoids across gut cell membranes; in addition, due to their scarce solubility in either water or organic solvents, flavonoids in the aglycone form are of appropriate molecular weight to permit their diffusion (Manach et al., 2004).

Bioavailability from a nutritionist's viewpoint is often expressed as the proportion of an ingested dose that is excreted in urine compared with the proportion excreted in feces over time (Birt et al., 2001). Flavonoids and isoflavonoids are present in foods as aglycone or, mostly, as glycosidic conjugates, but isoflavone glycosides have not been detected in human blood plasma or urine (Xu et al., 1994; Xu et al., 1995; King and Bursill, 1998). It is quite well established that once eaten, polyphenols enter a complex pathway of bio-transformation so that, the molecular forms reaching the peripheral circulation and tissues to be excreted are usually different from those present in foods (Manach et al., 2004).

The metabolism of several common polyphenols is now well understood. Metabolism of polyphenols occurs via a common pathway (Scalbert and Williamson, 2000). Aglycones can be absorbed from the small intestine. However, most polyphenols are present in food as esters, glycosides, or polymers that cannot be absorbed in their native form (Birt et al., 2001; Manach et al., 2004). These substances must be hydrolyzed by intestinal enzymes or by the colonic microflora before they can be absorbed. When microflora is involved, the efficiency of absorption is often reduced because it also degrades the aglycones and produces simple aromatic acid derivatives (Coureau et al., 2001; Gonthier et al., 2003). During the course of absorption, polyphenols are conjugated in the small intestine and later in the liver (Hollman and Katan, 1999; Birt et al., 2001; Manach et al., 2004).

With only a few exceptions the metabolism of dietary polyphenols leads to plasma conjugates (glucuronates or sulfates, with or without methylation) (Hollman and Katan, 1999), which means their total bioavailability is thus reflected in the amount of the parent compound plus all bioactive metabolites. In male rats,

for example, only a small portion of the aglycone has been detected free in the blood and in the urine after oral administration of the isoflavone daidzein; daidzein-sulfates were the major excretory products (10 fold more than the aglycone) (Bayer et al., 2001). These conjugates, which are more readily transported in the blood and excreted in bile or urine, are chemically distinct from their parent compounds, differing in size, polarity, and ionic form. Consequently, their physiologic behavior is likely to be different from that of the native compounds. It has been shown that glucuronidation reduces the biological activities of daidzein and genistein and the genistein sulfation decreases its antioxidant activity as well as its effect on platelet aggregation, inflammation, cell adhesion, and chemotaxis (Rimbach et al., 2003; Turner et al., 2004).

The relative concentration of different metabolites, in both plasma and tissues, is determined by the specific contribution of intestinal microflora, which could render the spectrum of conjugation products species- and gender-dependent. As an example equol, the microbial degradation products of the soy isoflavone daidzein formed in the gut (Setchell et al., 2002; Selvaraj et al., 2004), is abundantly produced in most animal models, whereas it appears to be produced in significant amounts only in subsets of humans (Birt et al., 2001). One of 6 men fed soy for several weeks was an equol producer, but 4 of 6 women fed soy for several weeks were equol producers (Lu et al., 1997). After feeding soy flour to 12 subjects for 3 days, equol production was inversely related to the production of *O*-desmethylangolensin (*O*-DMA), another daidzein metabolite produced during gut fermentation (Kelly et al., 1993). Several other urinary isoflavone metabolites were detected, and they were likely to have been derived from gut microbial fermentation, including 6-hydroxy-*O*-DMA, dehydro-*O*-DMA, dihydrogenistein, two isomers of tetrahydrodaidzein, and dihydrodaidzein (Birt et al., 2001). The health significance of these metabolites is unclear. However, in a breast cancer case-control study in Shanghai, breast cancer patients had lower urinary levels of the two major isoflavone metabolites, *O*-DMA and equol, as well as of the three major soy aglycones, in comparison with matched controls (Zhang et al., 1999). The affinity of the daidzein and genistein aglycones for ER are 10- and 40-fold higher, respectively, than those of the respective glucuronides, but the glucuronides still show weak estrogenic activity at physiologic concentrations (Zhang et al., 1999). Spencer and coworkers (2001) showed the inability of 5- and 7-*O*-glucuronides of epicatechin to protect fibroblasts and neuronal cells from oxidative stress *in vitro*, whereas epicatechin and methylepicatechin were protective. In a recent study, we indicated that some daidzein metabolites (*i.e.*, daidzein-7sulfate and equol) but not others (*i.e.*, daidzein-4'sulfate, daidzein-4',7sulfate, hydroxy-daidzein) retain the estrogen receptor-dependent potentially beneficial anti-tumor activity like their parent aglycone (Totta et al., 2005). Nevertheless, it is still difficult to draw any conclusions from the few existing studies regarding the effects of the type and position of conjugation on the various potential activities of polyphenols.

Polyphenol plasma concentrations vary highly according to the nature of the polyphenol and the food source. They are on the order of 0.3–0.75 mmol/L after consumption of 80–100 mg quercetin equivalent administered in the form of apples, onions, or meals rich in plant products (Hollman et al., 1997; Graefe et al., 2001). Naringenin in grapefruit juice appears to be absorbed even better: a peak plasma concentration of 6 mmol/L is obtained after ingestion of 200 mg (Manach et al., 2004). In contrast, plasma concentration of anthocyanins is very low, their action being thus restricted to the intestine (Cao et al., 2001). Isoflavones are the best absorbed flavonoids: plasma concentration of 1.4–4 mmol/L is obtained between 6 and 8 h in adults who consumed relatively low quantities of soy derivatives supplying 50 mg isoflavones (Xu et al., 1994; King and Bursill, 1998).

These reports open a pivotal issue in that the majority of studies published so far actually address a “misleading” matter by reporting the biological effect of isoflavone as they are ingested from food. Much work remains in order to determine the metabolic fate and bioavailability of specific flavonoids and isoflavonoids. The solubility, the metabolic fate of compounds, due to endogenous and exogenous biotransformation, and their interaction with other dietary components determine flavonoid bioavailability (Hendrich et al., 1998) and effects. Future research should open new avenues for the optimization of the effects of isoflavone administration and for the assessment of nutritional markers to evaluate the subject responsiveness to supplementation.

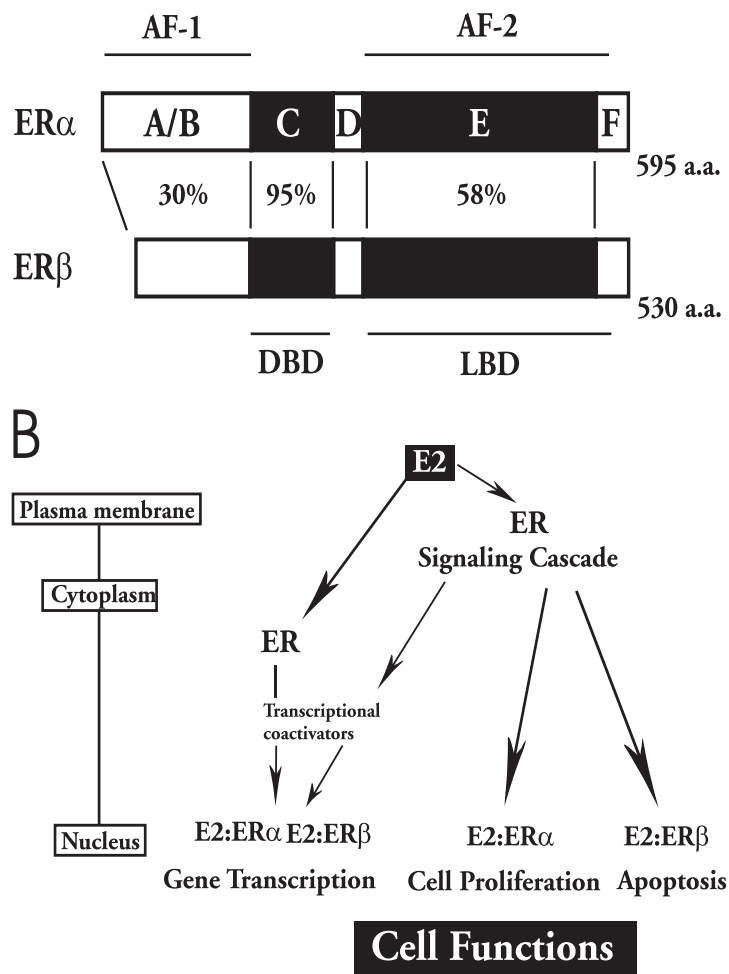
MECHANISMS OF ESTROGEN EFFECTS

The first hint of how steroid hormones work was the discovery of estradiol-binding proteins. The cDNA for ER was then identified in 1985 (Green et al., 1986), and later, a second receptor, ER β , was identified (Kuijper et al., 1996; Mosselman et al., 1996; Ogawa et al., 1998). ER α and ER β , encoded by two different genes, belong to the nuclear receptor superfamily (NR3A1 and NR3A2, respectively) of ligand-regulated transcription factors (Nilsson et al., 2001). Activated ERs largely bind to target promoter sequences as homo- or hetero-dimers (Marino et al., 2005).

ER α and ER β share an evolutionarily conserved functional structure (Figure 1A) consisting of the variable *N*-amino-terminal region involved in transactivation (A and B domains), the centrally located, well conserved DNA binding region (DBD, C domain), the hinge region involved in dimerization and in binding to heat shock protein 90 (D domain), the ligand (*e.g.*, E2) binding domain (LBD; E domain), involved in transactivation and synergic with the A/B region, and the *C*-terminal region (F domain) (Kumar et al., 1987; Enmark et al., 1997), which appears to play a role in modulating transcriptional activation by ER α (Peters and Khan, 1999). ER β is homologous to ER α at both LBD (58%) and DBD (95%), whereas the A/B region, the D domain, and the F region are not conserved (Matthews and Gustafsson, 2003). The two regions present in ERs that contribute to transcriptional activity are called activation functions (AFs). AF-1 is located in the *N*-terminal region and could be activated even in

a ligand-independent manner depending on the phosphorylation status of ER (Bunone et al., 1996; Weigel and Zhang, 1998; Tremblay et al., 1999); AF-2 is present at the *C*-terminus and shows a ligand-dependent activation (Weihua et al., 2003; Wrenn and Katzenellenbogen, 1993).

FIGURE 1: Domain organization of ER α and ER β (panel A) and schematic model illustrating the relationship between membrane, cytosolic, and nuclear actions of 17 β -estradiol (E2) on target cells (panel B). For details, see text.



Numerous mRNA splice variants exist for both ERs in both diseased and normal tissue. Hirata et al (2003) tentatively classified these splice variants into seven types. In type 1, different mRNAs are generated with the use of alternative transcription start sites. In type 2, one or more exons are skipped. In type 3, one or more exons are duplicated. In type 4, distinct mRNAs containing different 5'-untranslated exon(s) are synthesized. In type 5, distinct mRNAs possessing different coding exon(s) are generated. In type 6, mRNA is synthesized by intronic exons and coding exons 4/5-8. In type 7, mRNA with insertion of intronic exon(s) is generated. To date there is insufficient information as to whether or not all isoforms and splice variants of ER α and ER β described (Nilsson et al., 2001; Herynk and Fuqua, 2004) are expressed as

proteins or whether they have any major biological and physiological role. Zhang et al. (1996) examined the mRNA ratios of wild-type ER α to a number of exon deletion variants in 109 breast cancer specimens, and found that the expression of wild-type ER α was greater than the expression of any of the deletion variants in the majority of cases. ER β was identified much later than ER α , hence much less is known with respect to its splice variants, sequence mutations, and protein functions of these isoforms (see for review: Herynk and Fuqua, 2004).

E2 binding causes ER to dissociate from heat shock protein, to dimerize, to bind to specific DNA sequences (estrogen response element, ERE), and to stimulate the transcription of responsive genes. In established models of ER action, it was assumed that the activated ER-dimer manifests all its activities by interacting directly with ERE sequences within target genes. Several groups have shown that ER α and ER β form functional heterodimers *in vitro* and *in vivo* and that if both isoforms are expressed, the heterodimers predominate (Matthew and Gustafsson, 2003). However, the role of the ER α/β heterodimer in E2 signaling remains indefinite principally due to the presence of ER splicing variants (see above) that might produce heterogeneous population of ER dimers. Recently Monroe and co-workers (2005) reported that ER α/β heterodimers could regulate ligand-dependent gene expression of genes not regulated by either ER α or ER β homodimers. 102 genes uniquely regulated by the ER α/β heterodimer by E2 treatment, which are not regulated by either ER homodimer, have been described. The mechanisms underlying these effects are currently unknown and may involve the type of estrogen-responsive DNA element present in the promoter region(s) of these responsive genes (Monroe et al., 2005).

There is an accumulating amount of information indicating that ER α -regulated gene transcription, but not ER β (Matthew and Gustafsson, 2003), can occur also through the ER α indirect interaction with the transcription factors Sp1 and AP-1 (McDonnell, 2003; Nilsson et al., 2001). On the other hand, selective estrogen receptor modulator such as tamoxifen enhanced AP-1 mediated transactivation through both ER α and ER β (Maruyama et al., 2001). Monroe and co-workers (2005) suggested that the ER α/β heterodimer induces components of the AP-1 complex, possibly sensitizing the cells to tamoxifen-dependent transcriptional regulation through ER β , which may be one important physiological function of the ER α/β heterodimer.

Both in the direct and indirect action modes, the agonist-activated ER is not the transcription controller. In fact, ER needs to interact with a coregulatory proteins complex (coactivators or corepressors) at a short Leu-rich motif (*i.e.*, LeuXxxXxxLeuLeu) (Leers et al., 1998). This macromolecular complex provides a platform upon which additional proteins are assembled (McKenna and O'Malley, 2000; Smith and O'Malley, 2004). A large subset of these proteins, which possess chromatin-remodeling ability and tether, activated receptors to the basal transcriptional machinery has been described (Glass and Rosenfeld, 2000; Rachez and Freedman, 2001; Smith and O'Malley, 2004). The recruitment of cofactors with histone modifying and chromatin

remodeling activities by ERs overcomes the repressive features of chromatin leading to active transcription through the general transcriptional machinery (Kraus and Wong, 2002). Thus, the collaborative efforts of E2, receptor isoforms, ERE, and coactivators or corepressors can all contribute to the activation of genes in target tissues and organs (Nilsson et al., 2001; Matthews and Gustafsson, 2003; Smith and O'Malley, 2004).

The action mechanism described for E2 is based on its capability to modulate ER action by inducing conformational changes at LBD and to modulate coactivators binding. Natural ligand can coordinate the agonist and antagonist actions of ER by inducing different alterations within the ER structure (McDonnell et al., 1995; Wijayarathne et al., 1999). In the presence of the agonist E2 the crystal structures of ER α indicate that the binding site of the estrogen receptor can accommodate many ligands, which are planar, aromatic, and with 2 oxygen atoms spaced 11.5 Å apart (Jordan, 2003). The binding site (*i.e.*, LBD) is composed of amino acid residues belonging to α -helices 3, 4, 5, and 12. When, ER α -LBD forms complexes with E2, the binding site is capped by the H12 helical domain, thus preventing, the easy departure of the estrogen ligand and accounting for the very low K_d (100 pmol/L) of the E2:ER (Jordan, 2003; Barnes, 2004). Furthermore, the α -helix 12 positioned over the ligand-binding pocket forms an interaction surface for the recruitment of coactivators (Jordan, 2003; Brzozowski et al., 1997).

This 'genomic action' of steroid hormones occurs after a time-lag of >2 hours and explains some of their functions in physiological and pathological situations (Cidlowski, 2005).

This picture was challenged when a physiological dose of E2 was reported to increase the uterine cAMP level in ovariectomized rats within 15 seconds (Szego and Davis, 1967), an effect too rapid to be accounted for by genomic action. The action was not abrogated by transcriptional inhibitors and was termed 'rapid or nongenomic'. More recently evidence has started accumulating for such rapid effects being mediated by second-messenger systems that include mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), signal transducer and activator of transcription (STAT), epidermal growth factor receptor (EGFR), Src kinase, Shc kinase, protein kinase C (PKC), adenylate cyclase, GTP-binding proteins (G-proteins), and nitric oxide synthase (NOS) (Simoncini et al., 2000; Norman et al., 2004; Song et al., 2005; Levin, 2005; Manavathi and Kumar, 2005; Marino et al., 2005). The number of reports on rapid E2 effects is growing tremendously indicating that the action of E2 in living cells is mediated by various pathways rather than by a single uniform mechanism. On the basis of current findings, these rapid effects have been attributed in most cells to a population of ERs present on the plasma membranes (Pedram et al., 2002; Levin, 2005). We recently demonstrated that ER α undergoes S-palmitoylation on a cysteine residue (Cys447) present in the LBD which allows receptor anchoring to plasma membrane, association to caveolin-1, and which accounts for the ability of E2 to activate different signaling pathways (Acconcia et al., 2005a). The Cys399 residue present in the LBD of ER β seems also to be subjected to S-palmitoylation (M. Marino unpublished

data) suggesting that a similar mechanism may also work for ER β localization to the plasma membrane and association to caveolin-1. Thus, ER α and ER β have to be considered as a population of protein(s) which localization in the cell can dynamically change, shuttling from membrane to cytosol and to the nucleus on the dependence of E2 binding (Dan et al., 2003; Levin et al., 2005; Acconcia et al., 2005a). As a consequence, rapid and more prolonged E2 actions could be more finely coordinated.

The physiological significance of ERs-dependent rapid pathways is quite clarified, at least for some E2 target tissues. The mechanism by which E2 exerts proliferative properties has been assumed to be exclusively mediated by ER α -induced rapid membrane-starting actions (Marino et al., 1998; Castoria et al., 1999; Lobenhofer et al., 2000; Marino et al., 2001; Castoria et al., 2001; Marino et al., 2002; Marino et al., 2003; Acconcia et al., 2005b), whereas E2 induces cell death through ER β nongenomic signaling (Acconcia et al., 2005b). In the nervous system, E2 influences neural functions (*e.g.*, cognition, behavior, stress responses, and reproduction) in part inducing such rapid responses (Farach-Carson and Davis, 2003; Losel et al., 2003). In the liver, rapid E2-induced signals are deeply linked to the expression of LDL-receptor and to the decreased cholesterol-LDL levels in the plasma (Marino et al., 2001; Distefano et al., 2002). An important mode of E2-mediated atheroprotection is linked to E2 capability to rapidly activate endothelial NOS and NO production (Chambliss et al., 2002; Simoncini et al., 2000).

Finally, the capability of rapid E2-induced signal transduction pathways to modulate the classic genomic hormone mechanisms has been reported. Microarray analysis of gene expression in vascular endothelial cells treated with E2 for 40 min showed that some 250 genes were up-regulated; this could be prevented by Ly294002, a PI3K inhibitor (Pedram et al., 2002). Interestingly, the transcriptional activity of the E2-ER α complex could be inhibited by pre-treating cells with PD98059 and U0126, two ERK inhibitors (Acconcia and Marino, 2003). These findings support the idea that E2-induced rapid signals synergize with genomic events to maintain the pleiotropic hormone effects in the body (Figure 1B).

FLAVONOID ESTROGENICITY AND ANTIESTROGENICITY

As before reported, predating the discovery of ERs was the discovery that sheep fed mainly on red clover suffered reproductive dysfunction (Birth et al., 2001). This failure was determined to be due to the disruption of normal patterns of circulating steroid hormone concentrations in ewes grazing on clover. Both plasma E2 and progesterone levels were disrupted (Jacobs and Lewis, 2002). Later, these observations were understood to be the result of estrogenic activity of the clover isoflavones. This early study was later followed by many others, which evaluated the estrogenic activity of nutritional flavonoids. It has been reported that genistein and equol impair E2 and testosterone binding to human

sex hormone binding globulin relevant for hormone occurrence in the blood stream (Martin et al., 1996). Thus, genistein and other flavonoids could potentially affect clearance rates of sex steroid hormones and therefore the availability of the E2 to target cells (Martin et al., 1996; Oh et al., 2005). In 1997 the Organization for Economic Cooperation and Development (OECD) initiated a high priority activity to develop new and revised guidelines for screening and testing the estrogenicity of potential endocrine disruptors (OECD, 1998). One of the validated screening was the uterotrophic bioassay, which demonstrated the uterine growth of pre-pubertal or ovariectomized rats by following E2 or genistein treatment (*i.e.*, gavage or intraperitoneal injection) (Owens et al., 2003; Naciff et al., 2004; Ashby and Odum, 2004).

Nonetheless the estrogenic action mechanism of flavonoids emerged from being an agricultural oddity to having a biochemical basis only after the demonstration that they could apparently displace [^3H]-labeled E2 from the mammalian ER (Kuiper et al., 1998).

Due to activation of ER, these compounds were referred to as phytoestrogens because of their potential to disrupt estrogenic signaling. Note that, some plants contain steroidal estrogens, but they are not considered phytoestrogens by the strictest definition (Baker et al., 1999). The common phytosterols such as β -sitosterol, campesterol, and stigmasterol do not bind to human ER and do not exert estrogenicity in female rats (Baker et al., 1999). This allows for the consideration that the soy isoflavones genistein and daidzein, the chickpea isoflavone biochanin A, the clover isoflavone formononetin, the isoflavonoid-derived coumestran coumestrol, the flavonone naringenin, and the flavonol quercetin, all to be the major phytoestrogens relevant for human and animal health (Dixon, 2004).

In vitro studies indicate that ER α and ER β display marked differences in binding affinity and activation by some natural ER ligands including E2 (Kuiper et al., 1998; Escande et al., 2006). Although the affinity of flavonoids for the both ER α and ER β (as their aglycone forms) is lower than that of E2, competition binding studies performed by several authors confirmed that genistein, coumestrol, daidzein, and equol showed a distinct preference for ER β (Table 2; Shutt and Cox, 1972; Davis et al., 1998; Kuiper et al., 1998; Mueller et al., 2004; Escande et al., 2006). In comparison with genistein, 8-prenylnaringenin, a prenylated chalcone that occurs only in the hop plant, was found to be a 100 times more potent ER α agonist but a much weaker agonist of ER β in the estradiol-competition assay for receptor binding (Stevens and Page, 2004). Schaefer and co-workers (2003) claim that 8-prenylnaringenin is the strongest plant-derived ER α receptor agonist identified to date. In an *in vivo* study, the estrogenic potency of 8-prenylnaringenin was about 20,000-fold lower compared to E2 using uterotrophic and vagina growth assays (Stevens and Page, 2004). These distinctive estrogenic properties of 8-prenylnaringenin will foster new research on its potential as an ER modulator.

TABLE 2. Values of the dissociation equilibrium constant for ligand binding to ER α ($K_{ER\alpha}$) and ER β ($K_{ER\beta}$).

Ligand	$K_{ER\alpha}$ (nM)	$K_{ER\beta}$ (nM)	Ref.
17 β -Estradiol	0.04	0.11	Escande et al., 2006
Genistein	126	12.8	Escande et al., 2006
Coumestrol	80	27	Escande et al., 2006
Daidzein	262	85.3	Escande et al., 2006
Equol	13.3	3.6	Muller et al., 2004

ER α affinity order

17 β -Estradiol > Equol > Coumestrol > Genistein > Daidzein

ER β affinity order

17 β -Estradiol > Equol > Genistein > Coumestrol > Daidzein

E2 displacement by flavonoids could be equally well explained by flavonoids binding to a secondary site on the ER, thereby altering the K_d value of E2, rather than there being competitive binding between these compounds (Barnes, 2004). Verification that genistein binds to the active site of an estrogen receptor had to wait until 1999, when an X-ray diffraction crystal structure was reported for the ER α : genistein complex (Jordan, 2003). Later, the crystal structure for the ER β : genistein complex was resolved (Manas et al., 2004). As far we know these are the only crystal structures reported for nutritional flavonoid and no information is yet available on the other components of the flavonoid family or on their binding to ER β .

In addition, the crystal structure of the ER α -LBD: genistein shows that there are fundamental differences in the positioning of the α -helix 12 as compared with the agonist orientation induced by E2 (Jordan, 2003). Consistent with this data, genistein-bound ER α recruits the steroid receptor coactivator (SRC)-3 nuclear receptor (NR) box I of the p160 transcriptional coactivator family with less than half of the efficacy of E2-bound ER α , and SRC-3 NR box II is not recruited at all (Routledge et al., 2000; Bramlett et al., 2001). On the other hand, the crystal structure of ER β with genistein indicates that H12 is positioned on the surface of the LBD and the binding of SRC-3 NR box I showed a 3-fold amount in the presence of genistein greater than E2-bound receptor (Routledge et al., 2000; Bramlett et al., 2001). Thus, selective coactivator recruitment may be an underlying mechanism for tissue/cell (anti) estrogenicity of genistein. Although suggestive, these data are still pioneering and, in fact, more work in this field is requested to conclude that genistein could represent the paradigm for all flavonoid components; in addition more information about other coactivator or corepressor families is needed.

The further test of flavonoid (anti) estrogenicity is to measure the functional effect of isoflavones in cells expressing an ERE-luciferase reporter gene construct. Several investigators, including our group, reported that daidzein, genistein, naringenin, and quercetin increase the expression of luciferase in cells that express, coexpress, or even overexpress ER α or ER β (Mueller, 2002; Mueller et al., 2004; Virgili et al., 2004; Totta et al., 2004; Totta

et al., 2005). Whereas, flavonoids completely impair the interaction of ER α with the transcription factors Sp1 and AP-1 (Paech et al., 1997; Liu et al., 2002; Mattews and Gustafsson, 2003; Virgili et al., 2004). However, it is dangerous to make any presumptive statement about flavones from a single gene turned on or off by nutritional compounds.

During the past 20 years or so, molecular biology investigations have unraveled the genes that encode the proteins of life. Surprisingly, humans may have only about 24,000 genes (Pennisi, 2003). Levels of control of the use of gene information are expected to go beyond simple expression of the genes and their protein partners (Barnes, 2004). The benefit of sequencing the genome has been the engineering of DNA microarrays that essentially encompass the entire set of genes that are expressed in a given model. This has enabled investigators to examine the effects of a putative estrogen and not just the effects on their favorite gene or protein. Using MCF-7 cells, a significant number of genes responding to estrogen are found in a DNA microarray analysis and some of them were characterized (Teresaka et al., 2004). Among the genes related to tumor-associated genes, oncogenes and tumor-promoting genes are generally upregulated, whereas the genes related to tumor suppression and the ER α gene are downregulated (Inoue et al., 2002; Teresaka et al., 2004). This is consistent with the effects of estrogen, namely, the promotion of tumorigenesis (Fitzpatrick, 2003; Castagnetta et al., 2004; Yager and Davidson, 2006). For growth- and ion-associated genes and other genes, the expression of various transporters, synthetases, transcription factors, growth response genes, and structural genes was upregulated, indicating an enhancement of cell growth and proliferation (Teresaka et al., 2004). Meanwhile, the genes related to specific differentiation of the cell, such as those for neuronal proteins, were down regulated (Teresaka et al., 2004). Cluster analysis performed in the same cell line indicated that very similar profiles were obtained with 10 μ M genistein (Ramanathan and Gray, 2003; Naciff et al., 2004; Teresaka et al., 2004).

Naciff and coworkers (2002) sustaining that the gene expression changes are more sensitive than the classical uterotrophic assay for evaluating estrogenicity applied cluster analysis to the effects of genistein on the uterus of immature rats. 227 genes were affected by genistein, the majority of which were down-regulated (Naciff et al. 2004). Although genistein increased the expression of the progesterone receptor (an estrogenic effect), most of the remainder of its apparent effects in the developing uterus were not estrogenic. Several genes are involved in the homeostasis of calcium, consistent with genistein's properties as an agent for the prevention of osteoporosis (Naciff et al., 2002).

Such discrepancies are partially due to the analysis of gene array data which suffers from having many factors and few replicates, this leads to poor statistical power and most observed changes may be false (Barnes, 2004). Moreover, gene expression levels do not represent a whole cellular effect. A difference in gene expression profile cannot be immediately translated in a different cellular activity, since its phenotypic output at the cellular level is far to be determined. Gene arrays and other broad approaches in proteomics, metabonomics, and metabolomics are now

increasingly used to address the question of what an estrogen does and what a flavonoid does.

The most applicable and efficient approaches to estimate the effects of phytoestrogens are comprehensive analysis using DNA microarray and two-dimension polyacrylamide gel electrophoresis (2D-PAGE) (Garrels, 1989). Applying this method, Adachi and coworkers (2005) estimated the effects of daidzein in TM4 Sertoli cells. This analysis revealed that only the expression of 5 genes changed after daidzein exposure with respect to estradiol. The 5 genes were related to cell signaling, cell proliferation, and apoptosis suggesting a probable correlation with the inhibition of TM4 cell viability reported after exposure to daidzein (Adachi et al., 2005).

To facilitate the study of ligand-dependent ER genomic actions in reproductive and non-reproductive organs, the genome of a mouse model has been recently engineered with a transgene containing an exogenous reporter gene, luciferase, driven by an ERE-thymidine kinase promoter. This mouse model, named ERE-Luc, has proven to be a valuable tool for testing the effects of endogenous as well as exogenous estrogenic compounds (Maggi et al., 2004; Maggi and Ciana, 2005).

These methods are advantageous with respect to the competitive binding assay but more information is indispensable in order to obtain a classification of genes expressed in response to flavonoids, this will lead to a totally new picture of what flavones are. Waiting for these data it is now possible to conclude that some flavonoids share with E2 most of the gene expressed sustaining their estrogenicity.

It should however be pointed out that while the long-term estrogenic effects of phytoestrogens have been extensively studied, there is a lack of experimental data concerning the influence of natural estrogenic compounds on rapid E2-mediated mechanisms Belcher and Zsarnovszky, 2001; Maggiolini et al., 2004; Virgili et al., 2004; Totta et al., 2004; Klinge et al., 2005; Totta et al., 2005). The ability of these compounds to influence rapid actions of E2 in both reproductive and non reproductive E2-target tissues and how such effects may impact the normal development and physiological properties of cells is largely unknown. We recently demonstrated that quercetin and naringenin impair ER α -mediated rapid activation of signaling kinases (i.e., ERK/MAPK and PI3K/AKT) and cyclin D₁ transcription important for cell proliferation (Virgili et al., 2004). This effect was present only when HeLa cells, devoid of any ER isoforms, where transiently transfected with the human ER α expression vector demonstrating the ER-dependent mechanism. In the same cell system, naringenin activated the rapid phosphorylation of p38/MAPK and, in turn, the induction of a pro-apoptotic cascade (i.e., caspase-3 activation and PARP cleavage). Thus, naringenin decouples the ER α action mechanisms and thus preventing the activation ERK/MAPK and PI3K/AKT signal transduction pathways and driving cells to the apoptosis (Totta et al., 2004). Recent evidence favors the idea that besides coactivator association, the ER-LBD is essential and sufficient also for activation of rapid E2-induced signals (Marino et al., 2005). Thus, it is possible that flavonoids could induce different conformational changes of ER, also precluding the activation of rapid signaling cascades. In line with this hypothesis,

naringenin prevent ER α palmitoylation and reduce its association to membrane caveolin-1, impairing the activation of rapid signals (M. Marino unpublished results). On the other hand, naringenin does not impair the ER α -mediated transcriptional activity of an ERE-containing promoter (Virgili et al., 2004; Totta et al., 2004), this flavanone modulates specific ER α mechanisms and can be considered as "mechanism-specific ligands of ER" (Manolagas et al., 2002). The novel feature of these compounds is that they can selectively activate specific E2-signalling pathways in different organs, resulting in the beneficial effects of E2 in non-reproductive tissues without adverse effects to reproductive organs (Kousteni et al., 2002). Thus, naringenin, acting as a mechanism-specific ligand would function as ER α antagonists on certain pathways in all organs, eliciting the beneficial effects downstream of these pathways. In addition, some nutritional flavonoids (i.e., quercetin, naringenin, and daidzein) act as E2-mimetic in the presence of ER β rapidly activating p38/MAPK and the apoptotic cascade (Totta et al., 2004; Totta et al., 2005). Although the available evidence is currently limited, it seems probable that the nutritional compounds act as antagonist of ER-evoked rapid responses and agonist of ER β -dependent pro-apoptotic signaling.

CONCLUSIONS

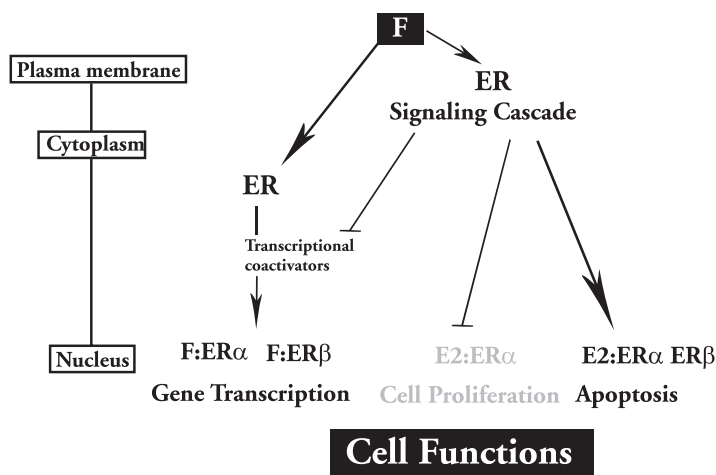
Over the past 20 years, researchers and food manufacturers have become increasingly interested in polyphenols. The chief reason for this interest is the recognition of their great abundance in human diet associated with the prevention of various diseases such as cancer, cardiovascular and neurodegenerative disease. In addition, this family of dietary components prevents the undesirable symptoms of menopause and may have protective roles in several E2-related diseases (i.e., osteoporosis and cardiovascular diseases in post-menopausal women) (Hertog et al., 1997; Kirk et al., 1998; Cassidy et al., 2000; Middleton, 2000; Kris-Etherton et al., 2002; Mikkola et al., 2002; Fitzpatrick, 2003; Dang and Lowik, 2005; Ricketts et al., 2005). Although interest in the potential benefits of flavonoid consumption continues to increase, the use of flavonoids in prevention of E2-related cancers or as a "natural" alternative to hormone replacement therapy remains controversial.

Owing to their basic chemical structure, the most obvious feature of flavonoids is in its ability to quench free radicals by forming resonance-stabilized phenoxyl radicals (Bors et al., 1997; Birt et al., 2001). Not necessarily related to its antioxidant capacity, and due to their high affinity for proteins, flavonoids have been reported to inhibit several kinases involved in signal transduction (Birt et al., 2001). For example, apigenin, kampherol, and genistein, at 25 μ M, reversed the transformed phenotype of v-ras-transformed NIH 3T3 cells that were associated with a reduction in phosphotyrosine content in the cells (Kuo et al., 1994). Gamet-Payraastre and coworkers (1999) recently reported that quercetin blocks particular isoforms of phosphoinositide 3-kinase or protein kinase C and their downstream-dependent cellular responses (Gamet-Payraastre et al., 1999). The inhibition by genistein (20–100 μ g/L) on *c-myc* oncogene expression in colon cancer cell lines has been suggested to be related to the

inhibition of tyrosine kinase activity (Heruth et al., 1995). Genistein (30–200 µg/L) has also reported to induce differentiation (Constantinou et al., 1990) and inhibit angiogenesis (IC₅₀ = 150 µM) (Fotsis et al., 1993).

At concentrations more physiologically achievable in the plasma (from 0.1 µM to 10 µM) after the consumption of meals rich in flavonoids (Manach et al., 2004), these compounds are thought to protect against degenerative diseases (*i.e.*, E2-related cancers) by regulating ER activity (Birt et al., 2001; Brownson et al., 2002; Totta et al., 2004). This has allowed us to enter a new era of understanding the mechanisms by which flavonoids exert their effects on human health. This era is characterized by an increased appreciation for the interacting network of responses that begin immediately upon exposure of cells to flavonoids and culminate in changes in gene expression affecting function and phenotype. The estrogenic effects of flavonoids originate from the activation of specific signaling pathways that modulate the membrane and nuclear actions of the dietary compounds. Understanding this potential for cross-talk within different pathways and the elaborate feedback mechanisms that are activated provides an opportunity to employ these chemicals as novel therapeutics that direct specific responses in target cells or that selectively modulate ER activity in specific target tissues and organs without interfering with ER activities in other tissues and organs. Figure 2 shows a model for ER signaling and cross-talk that provides a template for understanding the complex actions of flavonoids.

FIGURE 2: Schematic model illustrating the impact of nutritional flavonoids (F) on membrane, cytosolic, and nuclear actions of ERα and ERβ. For details, see text.



The field is moving ahead quickly with many new observations. The LBD of several nuclear receptors can accommodate flavonoids (e.g., PPARs, PXR, Ahr) expanding the effects of nutrition beyond the modulation of endocrine functions to the developmental and metabolic functions (Jacobs and Lewis, 2002; Shay and Banz, 2005). Another class of nuclear receptors, the estrogen receptor-related receptors (ERRs), deserves a particular mention. These orphan nuclear receptors share similarities of structure with ERs

although they do not bind estrogens (Ciguère, 2002). In spite of this, some flavonoids (e.g., genistein, daidzein) induced the activity of ERRα at concentrations that are comparable to those for the activation of ERα and ERβ (Suetsugi et al., 2003). It has been reported that ERRs and ERs have the potential to regulate common target genes and, in tissues where they are both synthesized, interfere or collaborate with each other to dictate the overall estrogenic response in a given cell type (Cheung, 2005). Major goals of future studies will be to identify flavonoid:ER and flavonoid:ERR target genes, using technologies such as chromatin immunoprecipitation assays and gene arrays, and to confirm the regulation of these genes by each receptor subtype in both cell- and animal-based models.

The challenges in the near future are to continue identifying the discrete actions of flavonoids and their metabolites on each intracellular pool of ER and to conclude the classification of flavonoid-dependent gene expression. Moreover, the ability of flavonoids to interact also with ER splicing variants and ERs heterodimers will further shed light on the power of these compounds to modulate the balance between proliferation and apoptosis of cancer cells in which these ER isoforms are most abundant (Zang et al., 1996; Herynk and Fuqua, 2004). Finally, considering the great variety of dietary flavonoids, it appears extremely unlikely that any one substance is responsible for all of the associations seen between plant foods and human health protection. The specific mechanisms of most flavonoids and isoflavonoids appear to be varied, complementary, and/or overlapping. Further investigations into the potential role of flavonoids and isoflavonoids in cancer prevention and/or therapy are warranted.

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